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**MOLECULAR BIOLOGICAL CHARACTERIZATION OF
AMPLIFIED ESTERASES FROM ORGANOPHOSPHATE
RESISTANT AND SUSCEPTIBLE
*CULEX QUINQUEFASCIATUS***

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ABSTRACT

Culex mosquitoes, as well as being vectors of filariasis and Japanese encephalitis, are a world wide biting nuisance. Organophosphorus insecticides (OPs) have been widely used to control *Culex* populations. Resistance to OPs has occurred and is typically mediated by the increase in non-specific esterase activity. The two esterases involved are classified as 'A' and 'B' esterases with respect to their preference for the substrates α - or β - naphthyl acetate. The commonest phenotype involves two elevated esterases, A₂ and B₂, which occur in complete linkage disequilibrium. The over expression of esterase B₁ is due to gene amplification.

Initially, in order to further study the molecular biology of OP resistance, full length cDNAs coding for both A₂ and B₂ esterases were isolated and sequenced from an OP resistant Sri Lankan strain of *Culex quinquefasciatus*, PelRR.

The B₂ esterase cDNA was isolated with PCR using primers sharing homology with the B₁ esterase cDNA and has 97.4% homology with esterase B₁ at the amino acid level. This confirmed that the B esterases belong to an allelic series. Partial genomic sequences of B₂ esterase from PelRR and four other OP resistant *Culex* strains were identical. This suggests that the initial B₂ esterase amplification has occurred only once. However, the cDNA sequence of a B₁ esterase cDNA isolated from an OP resistant Cuban strain of *Culex quinquefasciatus*, MRES, was different to that of the previously published B₁ esterase gene sequence. At the genomic level, the haplotype of the Cuban B₁ esterase gene, based on *Eco*RI endonuclease analysis, was also different, suggesting that the initial B₁ esterase gene amplification event has occurred at least twice. A B esterase cDNA from an

OP susceptible strain, PelSS, has also been partially sequenced. PelSS was derived from the same origins as PelRR but its B esterase cDNA sequence and haplotype of the gene are different. Thus, the B₂ esterase gene conferring OP resistance, as well as being amplified, is only found in the resistant strain, PelRR.

The A₂ esterase cDNA was isolated by screening a PelRR cDNA expression library with an anti-A₂ antiserum. The cDNA coded for a protein of 540 amino acids (the same as B₂ esterase) and shared 47% amino acid homology with B₂ esterase. This strongly suggests that the two genes arose from a duplication of an ancestral counterpart. Furthermore, screening of a PelRR genomic library with A₂ and B₂ esterase gene probes suggests that the two esterase genes, A₂ and B₂, are situated in tandem within the genome.

PCR was used to amplify the coding region of the PelRR A₂ esterase cDNA and this was co-transfected into the baculovirus expression system. The recombinant virus expressed an active A esterase.

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LIST ABBREVIATIONS

AChE	acetylcholinesterase
AcNPV	<i>Autographa californica</i> nuclear polyhedrosis virus
AMV	avian myeloblastosis virus
bp	deoxyribonucleotide base pair
BSA	bovine serum albumin
cDNA	deoxyribonucleic acid copy of messenger ribonucleic acid
cpm	disintegrations <i>per</i> minute
DEPC	diethyl pyrocarbonate
ddATP	2',3'-dideoxyadenosine 5'-triphosphate
DDE	1,1-dichloro-2,2-bis(<i>p</i> -chlorophenyl)ethylene
DDT	1,1,1-trichloro-2,2-bis(<i>p</i> -chlorophenyl)ethane
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide 5'-triphosphate
dT	2'-deoxythymidine 5'-triphosphate
EDTA	ethylenediaminetetra-acetic acid
ER	endoplasmic reticulum
<i>g</i>	gravitational force
GABA	gamma-amino butanoic acid
GST	glutathione S-transferase
IPTG	isopropylthio-β-D-galactoside
JH	juvenile hormone
kb	kilo deoxyribonucleotide base pair
kDa	kilo dalton
LINE	long interspersed repetitive element
LD ₅₀	concentration which gives 50% mortality
LT ₅₀	time which gives 50% mortality
LTR	long terminal repeat
mRNA	messenger ribonucleic acid
OP	organophosphorus insecticide
ORF	open reading frame
P450	cytochrome P450 mono-oxygenase
PAGE	polyacrylamide gel electrophoresis

Abbreviations

PCR	polymerase chain reaction
<i>Pfu</i>	<i>Pyrococcus furiosus</i>
pfu	plaque forming unit
pib	polyhedra containing inclusion body
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
rpm	revolutions <i>per</i> minute
SDS	sodium dodecyl sulphate
SSCP	single stranded chain polymorphism
<i>Taq</i>	<i>Thermus aquaticus</i>
TE	transposable element
TPI	triose phosphate isomerase
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
v/v	volume for volume
w/v	weight for volume
XDH	xanthine dehydrogenase

CHAPTER 1

CHAPTER 1: GENERAL INTRODUCTION

1.1 XENOBIOTIC RESISTANCE

Synthetic chemicals have been increasingly used as insecticides, herbicides and chemotherapeutic agents. They are used to treat bacterial, viral and parasitic infections as well as cancers and to control vectors of disease. Their extensive use has lead to a comparative increase in the tolerance of the target cells or organisms to the chemical. Since these chemicals are so effective, in time, they are bound to select for resistance. The consequence of this xenobiotic resistance is of great economic importance as well as a serious health risk. The pharmaceutical industry and research scientists alike are under great pressure therefore to overcome resistance as the range of available chemicals become increasingly useless in combating infection and controlling the vectors of disease. A clear understanding of the processes involved in resistance will obviously aid the production of new chemicals and hence research into the mechanisms of resistance must be maintained.

Over the past twenty years, research in the drug resistance of bacteria and the enormous breakthroughs in recombinant DNA techniques have significantly broadened our understanding of the molecular mechanisms of resistance. Drug resistance can be broadly classified as intrinsic (natural or *de novo* resistance) or acquired. In intrinsic resistance the organism, or cell in question, was resistant at the time treatment began. In acquired resistance the organism, or cell became resistant subsequent to the start of treatment. For a recent review see Hayes & Wolf, 1990.

Although an intrinsic resistance to novel chemicals seems at first surprising, it must be pointed out that all organisms are constantly under attack from a large number of foreign (xenobiotic) agents. During the course of evolution a basal resistance to all such challenges will have equipped the organism with defence against such attacks. Since most drugs have been developed recently, intrinsic resistance to such drugs is likely to have evolved independently. Such resistance will not necessarily be possessed by all members of a population due to polymorphic variation but it is likely to be widespread throughout the population. The mechanisms of intrinsic resistance are not always understood and reports simply state that a cell or organism does not respond to exposure to a chemotherapeutic agent. The most studied of intrinsic resistance mechanisms is that of the heat shock proteins (hsp's). The hsp's were originally discovered to be expressed when cells were put under hyperthermic stress and have since been shown to be expressed when cells are exposed to a range of other stresses. For a recent review see Schlesinger, 1994.

Acquired drug resistance describes the emergence of a resistant strain or cell line from a previously susceptible population. In many cases the resistance acquired is not solely towards the drug in question but may also be observed to a range of other chemicals, leading to cross-resistant strains.

1.2 ACQUIRED INSECTICIDE RESISTANCE

Organic synthetic insecticides such as the organochlorine DDT, were introduced in 1946 to control mosquito populations. In 1947, the first DDT resistant mosquito strains were seen in Florida. These were the salt marsh mosquitoes *Aedes taeniorhynchus* and *Aedes sollicitans* (for a historical review see Brown, 1986).

The four major groups of insecticides now being used worldwide are organophosphates (OPs), organochlorines (of which DDT is the most common), carbamates and pyrethroids. These groups of insecticides target the nervous system. The OPs and carbamates target acetylcholinesterase (AChE), which terminates the neurotransmission signal of acetylcholine by hydrolysis to acetate and choline. Inhibition of AChE by the insecticide therefore, causes repeated firing of neurones as the acetylcholine can not be hydrolysed. This leads to the death of the insect. The pyrethroids and some organochlorines (not the cyclodienes) target the sodium ion channels of the nervous system and the cyclodienes target the γ -amino butanoic acid (GABA) receptor, with similarly lethal results.

By 1992, 56 anopheline and 46 culicine mosquito species throughout the world were resistant to insecticides (WHO, 1992). Furthermore, multiple resistance to all insecticide groups in the same population has been seen in both anopheline and culicine species and since 1985, the number of new incidences of resistance in mosquitoes, whether of a country, species or insecticide has increased by 88 (WHO, 1992). The number of people suffering because of increased resistance is also on the rise. Today, over 250 million people infected with malaria and other vector borne diseases such as dengue are also on the increase both geographically and in terms of infected people (WHO, 1992).

The various biochemical mechanisms of insecticide resistance include reduced insecticide delivery, decreased insecticide influx, increased insecticide efflux, reduced insecticide metabolic activation, increased insecticide deactivation, insecticide sequestration, increased concentration of target sites, structural

alterations in target sites and increased repair of damaged target sites (Hayes & Wolf, 1990). The following mechanisms are those most commonly found in insects to confer resistance.

1.3 MECHANISMS OF INSECTICIDE RESISTANCE

1.3.1 Metabolic resistance

Metabolic resistance mechanisms account for most forms of insecticide resistance discovered to date and are caused by three main enzyme groups. They are the cytochrome P450 mono-oxygenases (P450s), the glutathione S-transferases (GSTs) and the carboxylesterases. These enzymes act by either sequestering or metabolizing the insecticide. Both actions prevent the insecticide from reaching its target in a sufficiently lethal dose. The catabolized insecticides are generally more hydrophilic than their parent compounds and this aids their removal from the insect.

1.3.1.1 *Esterases*

The esterase involvement in resistance has received the most attention of the three enzyme groups and hence the greatest advances have been made in this field. Generally, esterases are involved in resistance to OPs, carbamates and in some species, pyrethroids. Changes seen in the esterases can be broadly divided into quantitative or qualitative and both mechanisms prevent the insecticide from reaching its target site.

Resistant insects with qualitatively altered esterases show no apparent increase in the amount of esterase but an increase in the turnover of insecticide by an altered esterase. To date, malathion carboxylesterase is the only qualitatively

altered esterase known to be involved in insecticide resistance. In the house fly *Musca domestica*, the esterase has been shown to hydrolyse the carboxylester bonds of malathion (Matsumura & Hogendijk, 1964). The only other OP with carboxylester bonds is phenthoate and a malathion type esterase from a *Culex* strain has been shown to be associated with phenthoate resistance (Georghiou, 1969). This mechanism has also been seen in the mosquitoes *Anopheles stephensi* from Pakistan (Hemingway, 1982), *Anopheles arabiensis* from Sudan (Hemingway, 1983), *Anopheles culicifacies* from India (Malcolm & Boddington, 1989) and *Culex tarsalis* (Prabhaker *et al.*, 1987) where the resistance is associated with *w*, the white eye mutant (Calman & Georghiou, 1970). This type of malathion resistance has also been identified in the small brown planthopper *Laodelphax striatellus* (Sakata & Miyata, 1994), the two-spotted spider mite *Tetranychus urticae* (Matsumura & Voss, 1965) and the sheep blowfly *Lucilia cuprina* (Parker *et al.*, 1991). In *L. cuprina* the esterase E3, located in the microsomal fraction, is coded for by the OP resistance-related locus R_{OP-1} and maps to chromosome 4 (Hughes & Raftos, 1985). Native polyacrylamide gel electrophoresis (native PAGE) gels of homogenates of susceptible and OP resistant individuals showed that the E3 from the resistant strain was unable to catalyse the breakdown of α - or β -naphthyl acetate but the E3 from the susceptible strain could (Parker *et al.*, 1991).

The correlation between insecticide resistance and quantitative changes in esterase activity has been established using esterase substrates such as α - and β -naphthyl acetates. The correlation can also be substantiated by synergistic studies with carboxylesterase inhibitors such as DEF (*S,S,S*-tributyl phosphorothionate), IBP (*S*-benzyl *O,O*-diisopropyl phosphorothionate) and TPP (triphenyl phosphate) (Georghiou & Pasteur, 1978; Hemingway, 1982; 1983; Hemingway & Georghiou,

1984; Magnin *et al.*, 1988; Hemingway *et al.*, 1989b; Bisset *et al.*, 1990; Wirth *et al.*, 1990). These powerful inhibitors irreversibly bind to the active site of the esterase.

There are many reports of *Culex* strains which show that elevated esterase activity is associated with resistance. These include *Culex quinquefasciatus* (Georghiou & Pasteur, 1978; Hemingway & Georghiou, 1984; Raymond *et al.*, 1987; Magnin *et al.*, 1988; Bisset *et al.*, 1990; 1991; Hemingway *et al.*, 1990; Peiris & Hemingway, 1990b; Wirth *et al.*, 1990), *Culex pipiens* (Pasteur *et al.*, 1981a; 1981b; Villani *et al.*, 1983; Maruyama *et al.*, 1984; Fournier *et al.*, 1987; Villani & Hemingway, 1987), *Cx tarsalis* (Matsumura & Brown, 1961b; Apperson & Georghiou, 1975; Prabhaker *et al.*, 1987) and *Culex tritaeniorhynchus* (Takahashi & Yasutomi, 1987). Elevated esterase activity has also been associated with resistance in other insects including the peach-potato aphid *Myzus persicae* (Needham & Sawicki, 1971; Devonshire, 1977), the house fly *Musca domestica* (Kao *et al.*, 1985a; 1985b), the brown plant-hopper *Nilaparvata lugens* (Chen & Sun, 1994), the tobacco white fly *Bemisia tabaci* (Byrne & Devonshire, 1991; 1993), the cockroach *Blattella germanica* (Prabhakaran & Kamble, 1993; Hemingway *et al.*, 1993a; 1993b), the two-spotted spider mite *Tetranychus urticae* (Matsumura & Voss, 1964), the Egyptian cotton leafworm *Spodoptera littoralis* (Riskallah, 1983), the greenbug *Schizaphis graminum* (Siegfried & Ono, 1993; Siegfried & Zera, 1994), the citrus thrip *Scirtothrips citri* (Ferrari *et al.*, 1993), the tufted apple bud moth *Platynota idaeusalis* (Bush *et al.*, 1993) and the black fly *Simulium damnosum* (Hemingway *et al.*, 1989b).

1.3.1.2 Cytochrome P450 mono-oxygenases

P450s catalyse the reduction of molecular oxygen with the incorporation of one oxygen atom into the substrate to give an oxidised product. The P450 O-dealkylation of an insecticide increases its hydrophilicity and consequent efflux from the target cell. Insect P450s are found in many insect tissues throughout development, are present in multiple forms and are involved in the metabolism of insect hormones, secondary plant chemicals and insecticides (Ronis & Hodgson, 1989). In the malaria vector *Anopheles albimanus*, PCR with degenerative primers based on the insect CYP4 family of P450s revealed 17 different cDNAs (Scott *et al.*, 1995), demonstrating their substantial polymorphism. Involvement of P450s in resistance has been shown in the cockroach *B. germanica* (Siegfried *et al.*, 1990), the house fly *Musca domestica* (Hammock *et al.*, 1977; Ugaki *et al.*, 1985; Takahashi & Yasutomi, 1987; Scott & Lee, 1993), the fruit fly *Drosophila melanogaster* (Waters & Nix, 1988) and the mosquitoes *Anopheles subpictus* (Hemingway *et al.*, 1991) and *Cx quinquefasciatus* (Hemingway *et al.*, 1990). A full length P450 cDNA has been isolated and expressed from a resistant strain of *Musca domestica* (Feyereisen *et al.*, 1989) to study its role in resistance. The co-expression of a P450 (CYP6A1) from a resistant strain of house fly with house fly NADPH-cytochrome P450 reductase in *Escherichia coli* (*E. coli*) has been undertaken (Andersen *et al.*, 1994). This has shown that cyclodiene insecticides are metabolised by the CYP6A1 P450. Work on the P450-B subset of *D. melanogaster* has shown an increase in mRNA levels for the P450 in resistant flies (Waters *et al.*, 1992) and the resistance-associated locus has been mapped to chromosome III (Waters & Nix, 1988). The resistance-associated P450-B gene was smaller in size than its susceptible counterpart. Comparisons of the P450 genes from resistant and susceptible flies showed the

difference in size was caused by the presence of a transposable element in the gene from the susceptible fly, not present in the resistance-associated gene (Waters *et al.*, 1992). The transposable element contained AUUUA sequences and it has been shown that this sequence can lead to mRNA degradation (Shaw & Kamen, 1986). Thus, the absence of this sequence in the resistant strain was thought to be involved in the stability of the P450-B mRNA and therefore, the increased expression of the P450. However, further work on a large number of resistant and susceptible strains showed that the presence of the transposable element was not linked to resistance (Delpuech *et al.*, 1993).

Most OPs are used in the field in their phosphorothionate form. This form of the insecticide has low mammalian toxicity, is stable and due to its hydrophobicity, easily crosses the insect integument. Once inside the target cell, P450s convert the thionate to its oxon form, by the substitution of a sulphur atom for an oxygen atom. In *Musca domestica*, antiserum raised against P450 has been shown to inhibit the conversion of chlorpyrifos into its active oxon analogue, chlorpyrifos-oxon (Hatano & Scott, 1993), demonstrating the role of P450 in thionate to oxon conversion. The oxon analogues are extremely good inhibitors of the AChE target and these forms of the OPs are either hydrolysed or sequestered by resistant insects. For instance, the insecticidal effect of the oxon, malaoxon, compared to its thionate, malathion, on AChE in *Cx tarsalis* is 2000 times greater (Matsumura & Brown, 1961b).

1.3.1.3 Glutathione S-transferases (GSTs)

GSTs catalyse reactions in which the sulphur atom of glutathione provides electrons for nucleophilic attack on a second electrophilic substrate. The GSTs

cause resistance by the dehydrochlorination of DDT to DDE and the *O*-dealkylation of OPs (Hayes & Wolf, 1988). They can also cause the transfer of glutathione onto the insecticide thereby increasing its solubility and efflux from the cell.

High levels of GST activity have been detected in resistant insect strains of the house fly *Musca domestica* (Motoyama & Dauterman, 1975; 1977; Oppenoorth *et al.*, 1977, 1979; Ugaki *et al.*, 1985) and the mosquitoes *Aedes aegypti* (Grant *et al.*, 1991), *An. subpictus* and *Anopheles gambiae* (Hemingway *et al.*, 1991; Prapanthadara & Ketterman, 1993). GSTs from *Musca domestica* have been identified and purified (Fournier *et al.*, 1992b). Two classes of GSTs have been isolated, GST-1 and GST-2 and antisera prepared against the two classes show no cross-reactivity between the classes (Fournier *et al.*, 1992b). Each class appears to contain multiple forms with different pI values. A gene encoding *Musca domestica* GST-1 has been cloned and sequenced and shares a high level of sequence identity and immuno-reactivity with GST-1 from *D. melanogaster* (Toung *et al.*, 1990). In Cornell R, a resistant strain of *Musca domestica*, an increased level of mRNA and protein of the GST-1 when compared to the susceptible strain was seen (Fournier *et al.*, 1992b). This was not accompanied with an increase in signal at the genomic DNA level, suggesting that the increased GST-1 activity is caused by an increased level of transcription rather than a gene amplification.

A region of the *D. melanogaster* genome coding for the GST-1 family has been identified (Toung *et al.*, 1993) and members of this family, including DmGST-1 are expressed in adult flies. There is an amino acid identity between the GST-1 family of 53-75%. This is in contrast with the low level of homology between

DmGST-1 and DmGST-2 genes (Beall *et al.*, 1992). *In situ* hybridisation studies have shown that the two GST families are situated at distinct chromosomal loci (Beall *et al.*, 1992). Little is known of the control of expression of insect GSTs. However, genetic crossing experiments between a DDT resistant and wild-type strain of *Ae. aegypti* have shown that a *trans*-acting transcriptional repressor controls GST-2 expression (Grant & Hammock, 1992). The DDT resistant strain is thought to be homozygous for a non-functional repressor resulting in the elevated levels of the GST-2 seen in the strain.

1.3.2 Structural alterations in target sites

In many cases of insecticide resistance, the target site for the insecticide has altered to decrease the effect of the insecticide. In at least two target sites, this alteration is caused by a single amino acid change in the site's protein sequence. Obviously, the alteration in the target site must preserve its natural function.

1.3.2.1 Altered acetylcholinesterase (AChE) (EC 3.1.1.7)

AChE is the target site for the OPs and carbamates (Gepner *et al.*, 1978). The altered AChE enzyme is less susceptible to inhibition by the OPs and carbamates and hence can continue to turnover acetylcholine. This resistance has been seen in the house fly *Musca domestica* (Devonshire, 1975; Moores *et al.*, 1988), the fruit fly *D. melanogaster* (Fournier *et al.*, 1993), the aphids *M. persicae*, *Myzus nicotianae* and *Aphis gossypii* (Moores *et al.*, 1994; Suzuki & Hama, 1994; Silver *et al.*, 1995), the green rice leafhopper *Nephotettix cincticeps* (Iwata & Hama, 1972), the cattle tick *Boophilus microplus* (Nolan *et al.*, 1972), the Colorado potato beetle *Leptinotarsa decemlineata* (Zhu & Clark, 1995), the two-spotted spider mite *Tetranychus urticae* (Smissaert, 1964) and several mosquito strains including *Cx*

quinquefasciatus (Bisset *et al.*, 1990; 1991; Rodriguez *et al.*, 1993), *Cx pipiens* (Villani & Hemingway, 1987; Bonning & Hemingway, 1991; Rivet *et al.*, 1994), *Cx tritaeniorhynchus* (Hemingway *et al.*, 1986b; Takahashi & Yasutomi, 1987), *An. albimanus* (Hemingway *et al.*, 1984), *Anopheles nigerimus* (Hemingway *et al.*, 1986b) and *Anopheles sacharovi* (Hemingway *et al.*, 1985). The selection of this mechanism in *Culex* populations has always occurred in conjunction with and after the appearance of, the elevated esterase-based mechanism of resistance (Villani & Hemingway, 1987; Rodriguez *et al.*, 1993). The gene and protein for AChE have been well characterised in the fruit fly *D. melanogaster* (Gnagey *et al.*, 1987; Fournier *et al.*, 1992a). In insecticide resistant populations, the changes in AChE were seen at the level of translation, leading to increased AChE production to overcome the insecticide onslaught and also at the molecular level where a single point mutation altered the inhibition co-efficient (k_i) of the AChE for the insecticides (Fournier & Mutero, 1991; Pralavorio & Fournier, 1992; Fournier *et al.*, 1993). The point mutation led to one amino acid change, phenylalanine³⁶⁸ to tyrosine. The OPs and carbamates form a covalent bond with the active site serine of AChE and destroy its ability to hydrolyse acetylcholine. The binding isn't irreversible but the AChE is phosphorylated or carbamylated (in the case of OPs or carbamates respectively) and is only slowly regenerated over several hours. The resistance-associated AChE has a reduced affinity for the OPs/carbamates and effectively the insecticide no longer competes with the acetylcholine for the AChE. The mutated amino-acid is thought to be located in the active site of the AChE and be the cause of the low binding affinity of the insecticide (Mutero & Fournier, 1992). Other mutations of AChE that give increased OP resistance in *D. melanogaster* have also been detected and these include Phe¹¹⁵ to Ser, Gly³⁰³ to Ala and Ile¹⁹⁹ to Val (Pralavorio & Fournier, 1992).

1.3.2.2 *Altered γ -amino butanoic acid receptor (GABA receptor)*

The cyclodienes, members of the organochlorine family of insecticides, interact with the GABA receptor. An alteration in the GABA receptor, leading to insecticide resistance has been demonstrated in the fruit fly *D. melanogaster* (ffrench-Constant *et al.*, 1992; Steichen & ffrench-Constant, 1994; Bloomquist, 1994), the mosquito *Ae. aegypti* (Thompson *et al.*, 1993), the house fly *Musca domestica* (Anthony *et al.*, 1991), the cockroach *B. germanica* (Kadous *et al.*, 1983) and the red flour beetle *Tribolium castaneum* (Lin *et al.*, 1993). As for AChE, it has been shown that a single amino-acid change in the GABA receptor is responsible for cyclodiene resistance. It was demonstrated that the GABA_A receptor-chloride ionophore complex antagonists competed with the cyclodienes in vertebrate systems (ffrench-Constant *et al.*, 1991) and thus it was assumed that the cyclodienes interacted with a GABA_A type receptor in insect systems. This was shown in house fly brain (Deng *et al.*, 1991). Cyclodiene resistance in *D. melanogaster* was shown to be associated with a temperature sensitive phenotype, *Rdl* (dieldrin resistance) (ffrench-Constant *et al.*, 1993b) and chromosome walking at the *Rdl* locus lead to the isolation of the gene associated with cyclodiene resistance (ffrench-Constant *et al.*, 1991). The gene product showed high homology to the vertebrate GABA_A receptor and was the first isolated invertebrate GABA receptor. Sequencing of the GABA receptor gene from resistant and susceptible *D. melanogaster* populations showed that a single nucleotide mutation caused a single amino-acid change, Ala³⁰² to Ser, within the second membrane spanning region of the GABA receptor channel (ffrench-Constant *et al.*, 1993c). The Ala to Ser mutation was unequivocally shown to be the cause of the cyclodiene resistance using mutation studies in *Xenopus* oocytes (ffrench-Constant *et al.*, 1993a). Oocytes were injected with GABA

receptor mRNA from the susceptible insect and the receptor was functionally expressed and sensitive to the cyclodiene, dieldrin. However, when site directed mutagenesis was used to mutate the single nucleotide in the mRNA which gave rise to the Ala³⁰² to Ser mutation, this mRNA produced a functionally active GABA receptor in the oocyte that was insensitive to dieldrin (ffrench-Constant *et al.*, 1993a). The GABA receptor has also been cloned from resistant and susceptible *Ae. aegypti* populations and a similar nucleotide mutation leads to the same Ala to Ser amino-acid substitution in the resistant insect (Thompson *et al.*, 1993). The Ala to Ser mutation involved in cyclodiene associated resistance had also been shown in a further three insect orders; *Musca domestica* (Diptera), *Tribolium castaneum* (Coleoptera) and *Periplenta americana* (Dictyoptera) (ffrench-Constant, 1994), demonstrating a parallel evolution of the cyclodiene resistance mechanism.

1.3.2.3 Altered sodium ion (Na⁺) channel

The target site for the pyrethroids and the organochlorine DDT is the sodium ion channel. The resistance mechanism is known as '*kdr*' (knock down resistance) or '*super kdr*' (a level of resistance greater than that seen in '*kdr*') and in the house fly *Musca domestica* is caused by a reduced affinity of the target site for the insecticide (Ahn *et al.*, 1986a; Grubs *et al.*, 1988; Pauron *et al.*, 1989; Amichot *et al.*, 1992). Na⁺ channel insensitivity to insecticides has also been shown in the cockroach *B. germanica* (Dong & Scott, 1994) and the mosquitoes *Cx quinquefasciatus* and *Ae. aegypti* (Amin & Hemingway, 1989; Hemingway *et al.*, 1989a). In the house fly, nerve insensitivity to the pyrethroid, permethrin was shown to be recessive and crossing experiments suggested that the gene(s) responsible for resistance was (were) on chromosome III (Ahn *et al.*, 1986b). The

kdr mechanism was mapped to the Na⁺ channel gene in the house fly and a partial cDNA was isolated (Williamson *et al.*, 1993). This cDNA had a 99% homology with the *para* Na⁺ channel gene of *D. melanogaster* (Salkoff *et al.*, 1987). Using the house fly Na⁺ channel cDNA as a probe for RFLP analysis, different RFLP patterns were seen in house flies which were pyrethroid susceptible, those with the *kdr* mechanism and those with the *super kdr* mechanism (the *super kdr* mechanism was associated with two unique RFLP patterns). The RFLP patterns are evidence for different Na⁺ channel gene alleles. A second *D. melanogaster* Na⁺ channel gene, *DSCI*, has been cloned and sequenced from pyrethroid resistant and susceptible insects (Amichot *et al.*, 1992). A single nucleotide difference between the two leads to a single amino acid change. As in the case of the altered GABA receptor (MECHANISMS OF INSECTICIDE RESISTANCE 1.3.2.2), this change is likely to confer resistance.

1.3.3 Reduced insecticide delivery

Reduced insecticide delivery has been seen in *Ae. aegypti* (Matsumura & Brown, 1961a) and *Culex pipiens fatigans* (Stone & Brown, 1969). In these mosquitoes, a thickening of the cuticle greatly reduces the delivery of the insecticide to the target site. Cuticular thickening has also been shown in insecticide resistant *Musca domestica* house flies (Golenda & Forgash, 1989). Further reports have demonstrated that OP resistance in the Colorado potato beetle *Leptinotarsa decemlineata* is partly due to a reduced penetration of the insecticide (Argentine *et al.*, 1994). Cuticular thickening leading to reduced DDT delivery has been most studied in the house fly *Musca domestica*, where the penetration delaying factor (*pen*) was localised to chromosome III (Sawicki & Lord, 1970).

1.3.4 Behavioural changes

In Thailand, DDT is used for malaria control by house spraying. The mosquito *Anopheles minimus* has changed its preferred resting habitat from indoors to outdoors and this behavioural change reduces its contact with the insecticide (Bang, 1985). However, this is a contentious issue, since the *An. minimus* species is actually a complex and it is possible that the more endophilic species was wiped out by the DDT spraying.

1.4 ESTERASE CLASSIFICATION

Esterase is the collective term for biocatalysts that have a hydrolytic action on carboxylic acid esters and thus covers a wide range of enzymes. A classification introduced by Aldridge (1953a; 1953b) is still used today to distinguish these enzymes. Certain esterases are inhibited by paraoxon, while others are not. Esterases inhibited by 0.1 μM paraoxon are termed B esterases whilst those unaffected are termed A esterases. Studies have shown that the B esterases have an active site serine residue and thus are also termed serine esterases (and also serine hydrolases). The A esterases are thought to have an active site cysteine residue (Aldridge, 1993) and those which hydrolyse OPs are now more typically known as the 'phosphoric triester hydrolases' (EC 3.1.8). The B esterases are now known as the 'carboxylesterases' (EC 3.1.1.1) (Reiner, 1993; Walker, 1993). The exact physiological function of most carboxylesterases is not known and they are often referred to as 'non-specific esterases'. However they are known to be involved in the detoxication of xenobiotics and in fatty acid metabolism.

1.5 CARBOXYLESTERASES (EC 3.1.1.1)

1.5.1 Classification

In mammals the majority of research has been conducted on the carboxylesterases from rat, mouse, rabbit and human. Early research classified enzymes with esterase activity according to their substrate specificity. This method of classification was abandoned when it became apparent that the isolated proteins were of a non-specific nature, being able to hydrolyse a large number of carboxylic acid esters. Further work demonstrated that such proteins were carboxylesterases. These included porcine liver proline- β -naphthylamidase (Matsushima *et al.*, 1991), porcine pancreatic cholesterol esterase (DiPersio *et al.*, 1990), rat adipose tissue fatty acid ethyl ester synthase (Tsujita & Okuda, 1992), mouse egasyn (Medda *et al.*, 1986), rat liver monoacylglycerol lipase (also named palmitoyl-CoA hydrolase and aspirin-metabolizing carboxylesterase) (Mentlein *et al.*, 1985a), rat liver acyl-CoA thioesterase (Alexson *et al.*, 1993) and human monocyte-specific esterase (Scott *et al.*, 1992). Carboxylesterases from rat liver cells were separated by chromatofocusing and classified according to their isoelectric point (pI) (Gaustad *et al.*, 1992). The isolated esterases were named 6.4, 6.2, 6.0, 5.2 and 5.0. Esterases 6.4 and 6.2 were only found in Kupffer and endothelial cells whilst all the esterases were found in the parenchymal cells. The mammalian esterases are now classified according to their electrophoretic mobility in native PAGE. The slowest moving esterase is named ES-1 and the next as ES-2 and so on (Peters, 1982; Simon *et al.*, 1985).

In insects, most research has been carried on the carboxylesterases of *D. melanogaster*. Twenty-two soluble esterases have been identified by combining the

techniques of native PAGE and isoelectric focusing (Healy *et al.*, 1991). As for the mammalian system, they were named Est1 through Est22. The house fly *Musca domestica* carboxylesterases have been classified by both pI (4.8, 5.1, 5.3 and 5.6) (Kao *et al.*, 1985a) and electrophoretic mobility (EI through EVI) (Ugaki *et al.*, 1983), although no attempt has been made to equate the two systems. In the sheep blowfly *L. cuprina* 8 esterases have been identified using native PAGE (E1-4, E7-9 and E13) (Parker *et al.*, 1991). These were classified partly on the basis of previous work which identified 16 esterases, although not all from the same strain (Hughes & Raftos, 1985). E1 and E2 were classified as acetylcholinesterase and E3, 4, 9 and 13 as carboxylesterases.

In *Culex* mosquitoes, the carboxylesterases involved in insecticide resistance have been classified. Their classification is characterised both by their electrophoretic mobility in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and their preferential hydrolysis of α - and β -naphthyl acetate (Georghiou & Pasteur, 1978; Raymond *et al.*, 1987). Esterases that preferentially hydrolyse α -naphthyl acetate are termed A esterases while those that hydrolyse β -naphthyl acetate are termed B esterases. Obviously this can lead to confusion with the Aldridge (1953a; 1953b) classification. However all these *Culex* esterases are B esterases (carboxylesterases) by the Aldridge (1953a; 1953b) classification. The subscripts 1,2 etc. denote the mobility of the esterases in native PAGE, the slowest having the lowest numbered subscript. This system has largely broken down, since esterases with identical electrophoretic mobility have been given different names (Poirie *et al.*, 1992). Three major esterase phenotypes have been observed in resistant populations of the *Cx pipiens* complex. These are A₁ in Southern France and Italy (Pasteur *et al.*, 1981a; Severini *et al.*, 1993); B₁, mainly

in the Americas (Georghiou & Pasteur, 1978; Bisset *et al.*, 1990) and A_2/B_2 , which is the most common. The A_2/B_2 esterases are always co-elevated and found around the world (Villani *et al.*, 1983; Raymond *et al.*, 1987; Hemingway *et al.*, 1990; Wirth *et al.*, 1990; Peiris & Hemingway, 1993; Rivet *et al.*, 1994). However, studies on the haplotypes of the B_2 esterase genes from resistant populations have revealed that there are different B_2 esterase alleles for electrophoretically identical B_2 esterases (Poirie *et al.*, 1992). The peach-potato *M. persicae* also has elevated esterases and they are the only other insecticide resistance-associated elevated esterases that have been extensively studied. They are termed E4 and FE4 (E stands for esterase and F for fast, as FE4 migrates more rapidly than E4 on native PAGE) (Devonshire *et al.*, 1986b).

1.5.2 Physical properties

Nearly all carboxylesterases isolated to date have a monomeric molecular weight of about 60 kDa (Heymann, 1980) and some associate into oligomeric forms. However, the gene encoding a 23 kDa serine esterase from the cyanobacterium *Spirulina platensis*, has been characterised (Salvi *et al.*, 1994). Mammalian carboxylesterases are stable at neutral pH, have pI values ranging from 4.7-6.5 and are often glycosylated. ES-1 from mouse is a 65 kDa glycoprotein (Kadner *et al.*, 1992) and ES-29 is a 130 kDa dimer (Deimling & Gaa, 1992). Human monocyte-specific esterase is a trimer consisting of 63.4 kDa glycoprotein monomers with a pI range of 5.5-6.1 and a human carboxylesterase from alveolar macrophages is a trimer made up of 60 kDa monomers (Munger *et al.*, 1991). All the microsomal rat liver carboxylesterases are glycosylated (Robbi & Beaufay, 1986; 1988) and a carboxylesterase purified from rat lung was a trimer of 60 kDa monomeric subunits (Gaustad *et al.*, 1991).

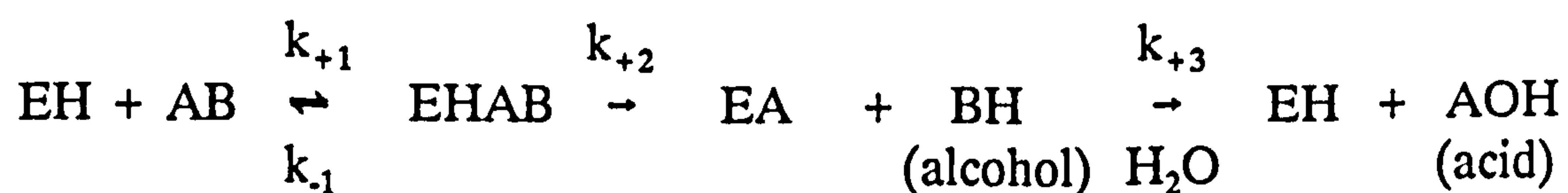
The molecular weights of the purified mosquito esterases A₂ and B₂ from *Cx quinquefasciatus* are 67 and 60 kDa respectively (Kettermann *et al.*, 1992; Jayawardena, 1992). Fournier *et al.*, (1987), reported a B₁ esterase of molecular weight 67 kDa and a dimeric A₁ esterase of 118-134 kDa. Typically the pI of carboxylesterases is between pH 4.7 and 6.5 (Heymann, 1980). In mosquitoes the pI of A₂ (Kettermann *et al.*, 1992), B₂ (Jayawardena, 1992), A₁ and B₁ (Fournier *et al.*, 1987) were 5.2, 5.1 and between 5 and 6 respectively. In *M. persicae*, the molecular weights of the two esterases E4 and FE4 were 65 kDa and 66 kDa respectively (Devonshire *et al.*, 1986b). pI values of purified house fly esterases range from 4.8 to 5.6 (Kao *et al.*, 1985a) and *D. melanogaster* juvenile hormone esterase has a pI of 5.4 (Campbell *et al.*, 1992). Two carboxylesterases purified from the termite *Odontotermes horni* have pIs of 5.4 and 5.6 and are dimers of 149 kDa (Sreerama & Veerabhadrapa, 1991).

The vertebrate carboxylesterase AChE exists as polymers of catalytic subunits. There are globular forms (G1, G2 and G4 which contain 1, 2 and 4 subunits, respectively) and asymmetric forms (ranging from 1 to 3 tetramers of subunits attached by disulphide bonds to a collagen-like tail) (Chatonnet & Lockridge, 1989). AChE from the Colorado potato beetle *Leptinotarsa decemlineata* is a 130 kDa dimer with a pI of 7.3 and AChE from the greater wax moth *Galleria mellonella* is a tetramer of 280 kDa (Habibulla & Newburgh, 1973). *D. melanogaster* AChE is a dimeric 165 kDa glycoprotein and the two active monomers are made up of two polypeptides of 18 and 55 kDa, resulting from the proteolysis of a 75 kDa precursor (Gnagey *et al.*, 1987). Studies on the *D. melanogaster* AChE show that the intersubunit disulphide bond which causes dimerisation of the two AChE monomers is cysteine 615 (Mutero & Fournier,

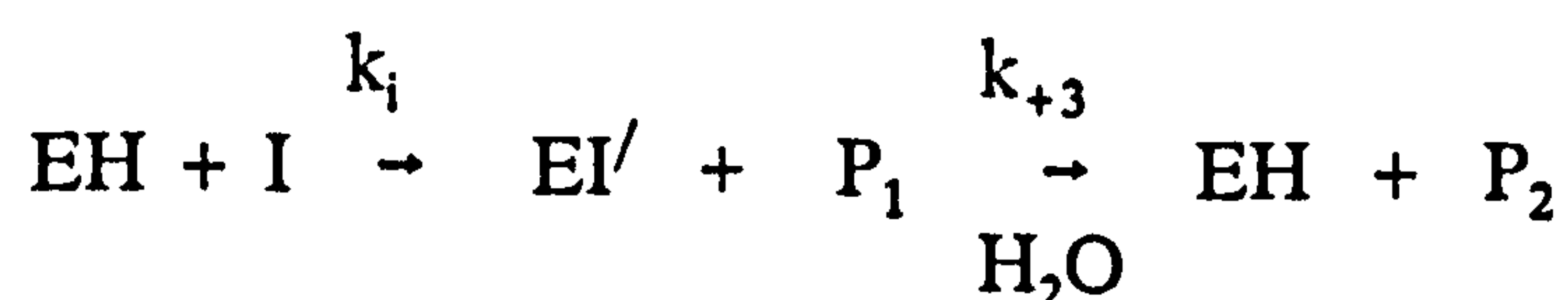
1992). Cysteine 615 has been mutated to an arginine residue and this single amino acid change prevented the formation of the AChE dimer. Similarly in the human AChE, a mutation of cysteine 580 to an alanine resulted in only monomeric AChE being secreted from recombinant cells (Velan *et al.*, 1991). This mutation did not affect the rate of catalysis or the affinity of AChE for its substrate acetylcholine. *D. melanogaster* AChE has four sites of asparagine linked glycosylation (Mutero & Fournier, 1992). This was determined by mutating asparagine residues 126, 174, 331 and 531. Comparisons of the mutant AChEs with the native enzyme on SDS PAGE showed that each mutation caused an apparent decrease in the molecular weight of the AChE when compared to the native AChE. This loss was due to the loss of the glycosylation site.

1.5.3 Chemical properties

In insecticide detoxification processes the carboxylesterases are able to catalyse carboxylester, carboxyamide ester and carboxythioester hydrolysis (Ahamad & Forgash, 1976). The carboxylesterases can also hydrolyse certain phosphoric acid esters. The accepted mechanism of esterase hydrolysis is given as:



or in the case of an esterase inhibitor:



EH is the enzyme, AB the substrate (or I the inhibitor), EHAB (the Michaelis complex, EA (or EI') the acylated enzyme, BH (or P₁) the alcohol product and AOH (or P₂) the acid product.

The rate of formation of the Michaelis complex (k_{+1}) is thought to be diffusion controlled and thus impossible to measure (Aldridge & Reiner, 1972). The inhibition co-efficient, k_i , is a measure of the affinity of the inhibitor (insecticide) for the enzyme (esterase). The biological substrate of an esterase typically gives rise to high k_{+2} and k_{+3} rates leading to rapid hydrolysis. The majority of carboxylesterases involved in OP and carbamate insecticide resistance have a very high affinity for the esterase and operate by sequestering the insecticide and thus the inhibition coefficient (k_i) is very small and the k_{+3} rate is very low. Sequestration has been shown for esterases B₁, B₂ and A₂ in *Cx quinquefasciatus* and esterase E4 in *M. persicae* (Devonshire, 1977; Devonshire & Moores, 1982; Kettermann *et al.*, 1992; Cuany *et al.*, 1993; Jayawardena *et al.*, 1994). The inhibition co-efficient, k_i , of four insecticides for purified A₂ esterase paralleled the cross-resistance spectrum of the *Cx quinquefasciatus* strains carrying this elevated esterase (Kettermann *et al.*, 1992). Cross-resistance is also seen in *M. persicae* where the E4 (and FE4) esterase is able to sequester not only OPs but also carbamates and rapidly hydrolyse the *trans*-isomer of the pyrethroid, permethrin (Sawicki *et al.*, 1978; Devonshire & Moores, 1982).

The exact physiological function of most non-specific carboxylesterases, as their name suggests, is still not clear. Their localisation in mammalian liver and lung implies a xenobiotic function. The microsomal carboxylesterases of the rat liver have been shown to catalyse the hydrolysis of a large number of physiological and

xenobiotic substrates (Mentlein *et al.*, 1980; 1984; 1985a; 1985b). Rat carboxylesterases are able to hydrolyse OPs (Maxwell, 1992) and the hydrolysis of the insecticides malathion, *trans*-permethrin and *cis*-permethrin has been demonstrated in rat and mouse microsomes (Soderlund *et al.*, 1982). In the mosquito *Cx quinquefasciatus*, preliminary studies have shown that esterases A₂ and B₂ have acylglycerol lipase activity, indicating a possible role in lipid-fatty acid metabolism as well as the defined role in detoxication of OPs (Ketterman *et al.*, 1992; Jayawardena, 1992).

The kinetics of AChE are the best defined of the carboxylesterases. AChE's catalytic mechanism is one of the most efficient known, substrate turnover has been shown to be 25000 molecules of acetylcholine per second and diffusion rate limited (Bazelyansky *et al.*, 1986). Catalysis was thought to involve electron transfer within a catalytic triad. The determination of the three-dimensional structure of *Torpedo californica* AChE (Sussman *et al.*, 1991) showed that a negatively charged glutamate residue was able to draw a hydrogen atom from an adjacent histidine, which in turn could draw a hydrogen atom from the active site serine. This enables nucleophilic attack on the substrate acetylcholine, resulting in the acylated enzyme. When this covalent bond is hydrolysed, acetate is released. The OPs and carbamates create a phosphorylated or carbamylated enzyme respectively, by a similar mechanism, which is hydrolysed at a very slow rate (Soreq *et al.*, 1990). In AChE, the catalytic triad is contained within the 'esteratic' site while the choline is held by what was known as the 'anionic' site. However, crystallographic data has shown that in the electric fish *T. californica*, the anionic site is in fact hydrophobic in nature (Sussman *et al.*, 1991). Mutation studies of human AChE have shown that Trp⁸⁶, in the hydrophobic site, is vital

for the binding of the quaternary ammonium ion of the substrate acetylcholine (Shaffman *et al.*, 1992; Ordentlich *et al.*, 1993).

1.5.4 Localisation and expression

In mammals the highest concentration of non-specific esterase activity towards aliphatic and aromatic substrates has been found in the liver, kidney and brain, where almost all the esterolytic activity at neutral or alkaline pH can be attributed to carboxylesterases (Kao *et al.*, 1985b). The carboxylesterases are predominantly found in the microsomal fraction of these tissues and the majority are associated with the rough endoplasmic reticulum. These include the five rat carboxylesterases previously discussed (Classification 1.5.1; Gaustad *et al.*, 1992), as well as ES-1 (Takagi *et al.*, 1988), ES-10A (Robbi & Beaufay, 1988) and ES-18 (Kluge *et al.*, 1990). In the rat, carboxylesterases are also located in the serum (Alexson *et al.*, 1994), adipose tissue (Tsuujita & Okuda, 1992), pancreas (DiPersio & Hui, 1993) and the lung (Gaustad *et al.*, 1991). In the mouse, carboxylesterases ES-8 is found only in the red blood cells, ES-16 only in the kidney and ES-5 only in the serum whilst ES-1, -2 and -5 are found in the plasma but not in red blood cells and ES-3, -7, -10 and -14 are found in the red blood cells but not in plasma (Peters, 1982).

Proteins that leave the cell have N-terminal amino acid signal sequences which direct the protein through the endoplasmic reticulum (ER) and from the cell. As mentioned above, most of the liver carboxylesterases are expressed within the ER and appear not to be exported, even though they possess signal sequences. A retention signal for soluble proteins of the ER was recently discovered which kept proteins with signal sequences in the lumen of the ER (Pelham, 1990). In the

majority of cases, the amino acid sequence KDEL at the carboxyl terminus of the protein ensured its retention, although further retention signals within the carboxylesterase family have also been determined and these include HVEL, HNEL, HTEL (Medda & Proia, 1992) and a carboxylesterase located in the ER of human alveolar macrophages which has the carboxyl terminal sequence HIEL (Munger *et al.*, 1991). This suggests that in the carboxylesterase family, HXEL (where X is any amino acid) is the minimum requirement for ER retention. Evidence of the requirement of the signal for ER retention was demonstrated in rat serum esterase (a secreted carboxylesterase) which was found to have a TEHT carboxyl terminus and hence, was not retained (Alexson *et al.*, 1994).

In insects, the esterases of *Drosophila* have been most studied and the location and expression of all *D. melanogaster* soluble esterases have been determined (Healy *et al.*, 1991). Est6 from *D. melanogaster* and EstS from *Drosophila virilis* are highly expressed in the anterior ejaculatory duct (Scott *et al.*, 1984) and the ejaculatory bulbs (Sergeev *et al.*, 1993) of the adult male respectively, although lower levels of Est6 expression are present elsewhere in both sexes of *D. melanogaster*. The expression of Est6 is located in newly laid eggs and rises until 36 hours after the adults eclose (Sheehan *et al.*, 1979). Deletion mutants of the 5' end of the Est6 gene have shown those regions to be necessary for the expression of Est6 in the adult male ejaculatory duct, salivary glands and respiratory system (Ludwig *et al.*, 1993). Newly eclosed females of *Drosophila simulans* and *Drosophila mauritania* show higher levels of expression of Est6 when compared to *D. melanogaster* and it has been shown that this is due to *cis* acting factors (Karotam & Oakeshott, 1993). *D. melanogaster* EstP and its homologues in *Drosophila pseudoobscura* (Est4) and *Drosophila buzzatii* (EstJ) are expressed

in similar ways but the homologues Est6, Est5 and EstI from the same three respectively, are not (Collet *et al.*, 1990). This has lead to the suggestion that EstP and its homologues are more essential than Est6 and its homologues (Collet *et al.*, 1990). Another carboxylesterase, AChE, from *D. melanogaster* is anchored in the plasma membrane and expressed almost exclusively in the head (Mutero & Fournier, 1992).

In *L. cuprina*, a detailed analysis of the expression of carboxylesterases has been undertaken (Parker *et al.*, 1991). The E1 and E2 AChE isozymes were expressed almost exclusively in the head. E1 was present in newly hatched larvae and its expression continued throughout adulthood, whereas E2 was not detected until early pupal stages. E3 was detected at high levels in the Malpighian tubules of all life stages and E4 levels were highest in the hemolymph. To date, no study has been undertaken to determine the localisation of the carboxylesterases (including those associated with insecticide resistance) in *Culex* mosquitoes.

1.5.5 Immunology

In the mouse, a multigene family of carboxylesterases are located on chromosome 8 and the esterases are divided into two distinct clusters (1 and 2) on the chromosome (Deimling *et al.*, 1983). Antiserum raised to an esterase of one cluster will not cross-react with antiserum from an esterase of the other cluster and the two clusters are thus immunologically distinct (Ronai *et al.*, 1985). However, antiserum from any esterase within a cluster will cross-react with all other esterases of that cluster, suggesting a homology between the esterases of each cluster.

A polyclonal antiserum raised against purified *Cx quinquefasciatus* A₂ esterase (Jayawardena, 1992) was used to probe a Western blot of a native PAGE gel containing both A₂ and B₂ esterases. The antiserum bound only to A₂. Similar results have shown there is no immunological cross-reactivity between the A and B esterases. Further more, when homogenates of the resistant *Cx quinquefasciatus* strain PelRR and the corresponding susceptible strain PelSS were Western blotted, the antiserum only visibly bound to the resistant strain, implying an increase in esterase protein in the resistant strain (Jayawardena, 1992). Similar results have been shown with the resistant *Cx quinquefasciatus* strain Dar91 when compared with susceptible strains Apo and Mon (Merryweather *et al.*, 1990). However, the use of a far more sensitive detection system has shown that the A₂ esterase antiserum will immunologically cross-react with the B₂ esterase and a number of vertebrate carboxylesterases, although the association is very weak (Karunaratne *et al.*, 1993b).

Antisera raised against esterases A₁ and B₁ from the OP resistant *Cx quinquefasciatus* strains S54 and TEM-R respectively showed increased protein production of 500-fold (B₁) and 70-fold (A₁) when compared with corresponding susceptible strains, thereby linking increased esterase production with increased insecticide resistance (Mouches *et al.*, 1987). The A₁ esterase antiserum cross-reacted with A₂ and A₃ esterases and the B₁ esterase antiserum cross-reacted with B₂ and B₃ esterases isolated from other *Culex* species (Mouches *et al.*, 1987; Beyssat-Arnaouty *et al.*, 1989). This demonstrates that within the two esterase classes, A and B, there exists a high a degree of homology. Homologues to the *Cx quinquefasciatus* A and B esterases do not exist in anophelines. This was demonstrated by a negative result in an ELISA using the *Cx quinquefasciatus* A

and B esterase antisera (Hemingway *et al.*, 1986a). However, with enhanced chemiluminescence, a more sensitive detection system, homologues of *Cx quinquefasciatus* A esterases have been detected in anophelines (Karunaratne *et al.*, 1995). The antiserum raised against the *M. persicae* E4 carboxylesterase was used to demonstrate that resistant aphids had increased amounts of E4 and a similar pattern was also shown to exist in the aphid *Phorodon humuli* (Devonshire *et al.*, 1986a; French-Constant & Devonshire, 1988).

1.5.6 Genetics

The inheritance and linkage relationships in mammalian esterases have been well documented for the mouse, rabbit, rat and human. There are many loci for carboxylesterases and polymorphism is common. In the mouse, most loci have at least two alleles (Peters, 1982; 1984; Eisenhardt & Deimling, 1983; Deimling *et al.*, 1983; Berning *et al.*, 1985; Medda *et al.*, 1986; Deimling & Gaa, 1992). Most of the carboxylesterase genes are found on chromosome 8 and are arranged in two clusters, 6.9 centimorgans apart (Peters, 1982; Ronai *et al.*, 1985). As mentioned before, the two clusters of genes are immunologically distinct and probably arose from an initial gene duplication and subsequent duplications within the cluster (Ronai *et al.*, 1985). Similar clusters are seen in other mammals. Human carboxylesterase clusters map to chromosome 16 (Kroetz *et al.*, 1993), rabbit clusters to chromosome 6 (Zutphen *et al.*, 1987) and rat to chromosome 5 (Alexson *et al.*, 1994). The similarity between rat ES-1 and many other carboxylesterases has led to the hypothesis that an ancestral esterase gene existed before the divergence of the vertebrates and invertebrates (Takagi *et al.*, 1991).

In the house fly *Musca domestica* (and in other insects), insecticide resistance (due to decreased uptake of insecticide, target-site insensitivity and altered metabolic activity) was shown to be an inherited characteristic (Oppenoorth & Van Asperen, 1960; Plapp, 1984). In *An. stephensi*, the locus for malathion resistance was shown to be 24 cross-over units from the dieldrin resistance-associated locus and 30 cross-over units from the *diamond palpus* mutant (Rowland, 1985). In *Cx tarsalis*, malathion resistance was linked to the *white eye* recessive marker (Calman & Georghiou, 1970). The genetics of two esterase loci in adult *Cx pipiens* mosquitoes have been studied by starch gel electrophoresis (de Stordeur, 1976). The esterases coded for by the two loci, named Est-1 (three alleles) and Est-2 (eight alleles), preferentially hydrolysed α - and β -naphthyl acetate respectively. The loci were shown not to be sex linked but they were linked to each other, with a cross-over frequency of 8.6%. In an OP resistant strain of *Cx pipiens*, very strong esterase bands (as visualised in starch electrophoresis) were seen and these were shown to be inherited as semi-dominant characteristics (Maruyama *et al.*, 1984). The esterases (A and B) involved in *Cx quinquefasciatus* OP resistance have been attributed to two closely linked gene loci, Est-3 and Est-2 respectively (Pasteur *et al.*, 1981a; 1981b) and have been shown not to be sex linked (Georghiou *et al.*, 1980; Peiris & Hemingway, 1993). In *Cx quinquefasciatus*, esterases A₄ and B₄ are under the control of two very tightly linked loci, approximately 0.8 centimorgans apart (Poirie *et al.*, 1992). Furthermore, crosses of *Cx quinquefasciatus* strains with either the B₁ esterase phenotype or the A₂/B₂ esterase phenotype show that these characters behave as independent loci (Wirth *et al.*, 1990) and the amplified B₁ esterase genes segregate as a block (Ferrari & Georghiou, 1991).

Mosquitoes have three pairs of chromosomes and inheritance patterns have shown that the resistance-associated esterase genes are on chromosome II for *Cx tritaeniorhynchus* (Takahashi & Yasutomi, 1987) and chromosome III for the *Cx pipiens* complex (Pasteur *et al.*, 1981a). The amplified B₁ esterase gene of *Cx quinquefasciatus* has also been tentatively assigned to a cluster on chromosome II (Nance *et al.*, 1990). However, amplification of the B₁ esterase gene could have altered chromosome III to such an extent that it was incorrectly assigned as chromosome II. The qualitatively altered esterase gene (E3) associated with malathion resistance in the sheep blowfly *L. cuprina* is on chromosome 4 (Hughes & Raftos, 1985). In the aphid *M. persicae*, the elevated levels of E4 esterase are associated with resistance (Needham & Sawicki, 1971; Devonshire, 1977). The E4 esterase gene is amplified (Field *et al.*, 1988) and an A1,3 (from chromosome 1 to 3) gene translocation is seen in very resistant insects (Devonshire & Sawicki, 1979). *D. melanogaster* Est6 maps to chromosome 3 (Scott *et al.*, 1984), whilst in the sibling species *Drosophila mojavensis* and *Drosophila arizonensis*, esterases Est2, 4 and 5, all map to chromosome 2 (Zouros *et al.*, 1982). Est2 mapped 34.7 +/- 2.94 centimorgans from Est4 and Est5. Since Est4 and Est5 were calculated at being only 0.16 recombination units apart and products of the alleles of the two loci form heterodimers, it was suggested that the two genes arose from a recent duplication (Zouros *et al.*, 1982). Gene duplication has also been predicted in *D. melanogaster* (Est6 and EstP) and their homologues in *D. pseudoobscura* (Est5 and Est4) and *D. buzzatii* (EstI and Est J) (Collet *et al.*, 1990; Brady *et al.*, 1990).

1.5.7 Molecular biology

The advent of recombinant DNA techniques has increased our understanding of biological systems. The gene (or cDNA) sequences of many carboxylesterases

(non-specific and those of a known physiological role) are now available from a diverse range of organisms. Gene (or cDNA) sequences of esterases with a known function include juvenile hormone esterase from the tobacco budworm *Heliothis virescens* (Hanzlik *et al.*, 1989) and AChE from *D. melanogaster* (Hall & Spierer, 1986; Fournier *et al.*, 1989), *An. stephensi* (Hall & Malcolm, 1991), the electric fish *T. californica* (Schumacher *et al.*, 1986) and human (Soreq *et al.*, 1990). Non-specific esterase gene (or cDNA) sequences include those from rat (Takagi *et al.*, 1988; 1991; Robbi *et al.*, 1990), human (Riddles *et al.*, 1991; Munger *et al.*, 1991), the aphid *M. persicae* (Field *et al.*, 1993), *Cx quinquefasciatus* (Mouches *et al.*, 1990) and *D. melanogaster* (Cooke & Oakeshott, 1989; Brady *et al.*, 1990; Collet *et al.*, 1990). Homologues of the non-specific esterases have been found in a variety of mammals. Using RFLP analysis, the ES-1 gene from mouse was shown to have homologues in human, monkey rat and rabbit (but not chicken) (Kadner *et al.*, 1992) and a human esterase cDNA has been isolated by PCR, based on the known sequence of rabbit and rat carboxylesterase cDNAs (Riddles *et al.*, 1991).

It was proposed that the carboxylesterases belonged to a large family of enzymes with a charge relay catalytic triad consisting of an active site serine nucleophile, along with aspartic acid and histidine residues (Myers *et al.*, 1988). Since then, the three dimensional atomic structure for the electric fish *T. californica* AChE has been deduced (Sussman *et al.*, 1991). The AChE was shown to have a narrow active site gorge which reached half-way into the protein and OPs were shown to form a covalent bond with serine²⁰⁰, demonstrating the serine as the active site nucleophilic residue. Mutation of the serine to a cysteine showed a decrease in activity but the enzyme was able to function as a cysteine hydrolase (Cole *et al.*, 1993). Mutation studies confirmed that the catalytic triad of human AChE consists

of Ser²⁰³, Glu³³⁴ (not Asp, as had been suggested; Myers *et al.*, 1988) and His⁴⁴⁷ (Shafferman *et al.*, 1992). In rat cholesterol esterase, Ser¹⁹⁴ and its homologue in pig cholesterol esterase was shown to be the active site nucleophile (DiPersio *et al.*, 1990).

The carboxylesterases belong to the α/β hydrolase fold enzymes (Ollis *et al.*, 1992). This fold is common to several hydrolytic enzyme groups which differ widely in function and phylogenetic origin and include carboxylesterases, carboxypeptidases, diene lactone hydrolases, dehalogenases and lipases. The core of each enzyme is similar: an α/β sheet of eight β -sheets connected by α -helices. The active site nucleophile is always located between β -strand 5 and α -helix C. The acid member of the triad (Glu or Asp) is on a loop following β -strand 7 and the third member of the triad, the histidine, at the end of β -strand 8 (Ollis *et al.*, 1992). The discovery of the α/β hydrolase fold, added to the three previously described groups of enzymes with catalytic triads: the eukaryotic serine proteases, the cysteine proteases and the subtilisins. Of all the enzyme structures known, about 10% conform to the eight stranded α/β domain, indicating the evolutionary importance of this catalytic structure (Farber & Petsko, 1990).

An analysis of the relationship between sequence conservation and the three-dimensional structure in a large family of carboxylesterases, lipases and related proteins has been undertaken (Cygler *et al.*, 1993). The alignment was based on the knowledge of the three-dimensional structures of *T. californica* AChE (Sussman *et al.*, 1991) and a lipase (EC 3.1.1.3) from the fungus *Geotrichum candidum* (Schrag & Cygler, 1993). These X-ray structures showed the exact position of the α -helices and β -strands in the enzymes and enabled the other

sequences to be accurately superimposed on the alignment. The deduction of the active site triad residues by mutation for a number of carboxylesterases was confirmed by this alignment. The serine nucleophile is conserved throughout the esterases and lipases, as is the histidine. However, the acid residue could be Asp or Glu. An Asp residue was seen in mammalian lipases, cholinesterases and *Drosophila* carboxylesterases. To date, all other carboxylesterases have a Glu acidic residue. The alignment also showed 24 invariant residues and 49 well conserved residues in 29 of the sequences.

In both *M. persicae* and *Cx quinquefasciatus*, the elevation of esterases that sequester the OPs have been shown to be the cause of resistance (Needham & Sawicki, 1971; Curtis & Pasteur, 1981; Peiris & Hemingway, 1990a). Elevation of a protein can be due to either stabilisation of the mRNA coding for the protein, increased transcription and/or translation of the gene and mRNA respectively, or by gene amplification. In *Cx quinquefasciatus*, a partial cDNA, which coded for a polypeptide that reacted to an elevated B₁ esterase antiserum from an OP resistant laboratory strain (TEM-R) of *Cx quinquefasciatus* was isolated (Mouches *et al.*, 1986). This cDNA was used to probe *Eco*RI digested genomic DNA from TEM-R and an insecticide susceptible strain, S-Lab. The result showed a huge increase in signal in the TEM-R strain, compared to that of S-Lab. This suggested that gene amplification was responsible for the elevated esterase activity and consequently OP resistance in the *Cx quinquefasciatus* TEM-R strain. The same cDNA probe was used to show that gene amplification, to differing degrees, was also present in natural populations of OP resistant *Cx quinquefasciatus* (Raymond *et al.*, 1989). In TEM-R, the level of gene amplification was estimated to be about 250-fold (Mouches *et al.*, 1986).

The genomic DNA sequence and predicted amino acid sequence of the *Cx quinquefasciatus* TEM-R B₁ esterase has been reported (Mouches *et al.*, 1990). The B₁ esterase is composed of 540 amino acids and based on the alignment of esterases mentioned above, the triad of catalytic residues were assigned to Ser¹⁹¹, Glu³²⁴ and His⁴⁴². The gene sits within an amplification unit, or 'amplicon', which has a 25 kb core within at least 30 kb (Mouches *et al.*, 1990). The gene is framed by highly repetitive sequences within the amplicon and these are thought to be involved in amplification, although very little is known about this process at present. Using the B₁ esterase cDNA probe, RFLP patterns of other B esterases have been determined (Raymond *et al.*, 1989; 1991). The ability of a B₁ esterase cDNA probe to hybridise under high stringency to amplified DNA from *Cx quinquefasciatus* strains expressing different B esterases, implied that the B esterases are allelic. Those mosquito strains expressing a B₁ esterase always had the same B esterase gene restriction map as did those expressing a B₂ esterase (although the pattern was different to the B₁ esterase gene). This suggested that all the B₁ genes are identical, as are all the B₂ esterases genes. Since amplified B₂ esterase genes are found worldwide, this has lead to the theory that the B₂ esterase gene amplification occurred only once and the amplified genes have since spread around the world (Raymond *et al.*, 1991). However, more recently, two amplified populations of B esterase genes (known as B₄ and B₅), whose products were electrophoretically identical to B₂ esterase, were shown to have different RFLP patterns to each other and to B₂ esterase (Poirie *et al.*, 1992).

Amplified esterase genes have also been shown to be the underlying mechanism of OP resistance in *M. persicae* (Field *et al.*, 1988). cDNAs for the two elevated esterases, E4 and FE4, have been isolated (Field *et al.*, 1993). The E4 and FE4

cDNAs code for proteins of 529 and 541 amino acids respectively. There are only 20 nucleotide differences between the two cDNAs which lead to only 9 amino acid differences in the mature proteins. The active site triad residues were assigned to Ser²⁰⁰, Glu³²⁷ and His⁴⁴⁰. Only one E4 type esterase gene has been isolated from a susceptible *M. persicae* population and its sequence identity with both E4 and FE4, suggest that a recent gene duplication event lead to the appearance of both E4 and FE4 genes in resistant populations (A.L. Devonshire, personal communication). A *M. persicae* population with only low OP resistance levels was put under insecticide stress and as resistance increased, so did the number of amplified copies of the E4 gene and mRNA levels (Devonshire & Sawicki, 1979), doubling between each generation of aphid that showed higher resistance levels. RFLP analysis showed that the esterase genes of those aphids with very high resistance levels had been translocated from chromosome 1 to 3 (A1,3) (Field *et al.*, 1989). Further studies showed that the very resistant aphids could lose their resistance if selection pressure was not upheld (ffrench-Constant *et al.*, 1988). The loss of resistance was due to a decrease in esterase activity brought about by a decrease in mRNA levels of E4 esterase but not a decrease in amplified DNA (and therefore in gene copy number) (Field *et al.*, 1989; Sawicki *et al.*, 1980). It was then demonstrated that methylation of two *MspI* restriction sites in the second exon of the E4 gene was essential for its expression (Field *et al.*, 1989; 1993) and these sites were not methylated in the aphid population that had lost its resistance. Reselection of the susceptible populations caused a rapid return to the high resistance levels and methylation of the *MspI* sites (Field *et al.*, 1989).



1.6 BACKGROUND AND AIMS OF THE PRESENT STUDY

The use of OP insecticides against the mosquito *Cx quinquefasciatus*, a vector for filariasis and Japanese encephalitis, has resulted in the emergence of OP resistant populations throughout the world. Resistance is due to the elevation of esterases and the two commonest phenotypes are B₁ esterase and A₂/B₂ esterases. To date, the A₂ and B₂ esterases have always been seen to be co-elevated. The overproduction of the B esterases is due to gene amplification and the gene sequence for esterase B₁ and its predicted amino acid sequence has been deduced (Mouches *et al.*, 1986; 1990; Raymond *et al.*, 1989). It has been suggested that few esterase gene amplification events have taken place and the products of these have recently spread worldwide through migration (Raymond *et al.*, 1991). The assumption that amplified genes can invade populations so quickly has important implications in mosquito control. Therefore the molecular characterisation of amplified esterase genes is essential to assess the number and migration rate of individual amplified genes.

The aims of this study are thus:

To determine how common the phenomenon of gene amplification is in *Cx quinquefasciatus* OP resistance. To try and answer this question it will first be important to isolate and sequence a B₂ esterase gene and compare it with the previously isolated B₁ esterase gene in order to determine if the two genes have been amplified separately. The comparison at the nucleotide level of B₂ genes from around the world to determine their similarity to one another and thus the probability that they all arose from a single amplification event also needs to be addressed. The isolation and sequencing of an A₂ esterase gene and its

comparison to the B esterase genes will demonstrate the similarity between the two genes and may help in the understanding of why both genes are over expressed. It is also important to determine if the A₂ esterase gene is amplified and its chromosomal location with reference to the B₂ esterase gene. If the two genes are co-amplified on the same amplicon, then the question of why this phenotype is so common also needs to be addressed. If esterase gene amplification and migration is occurring more frequently than at first imagined, then the occurrence and spread of OP resistance will pose a far bigger problem than at first imagined.

CHAPTER 2

CHAPTER 2: ISOLATION OF THE FIRST FULL LENGTH CDNA CODING FOR B₂ ESTERASE FROM AN OP RESISTANT STRAIN OF *CULEX QUINQUEFASCIATUS* (PELRR) AND ITS HOMOLOGY WITH OTHER B ESTERASES

This CHAPTER describes the isolation, by PCR techniques of the first full length B₂ esterase cDNA. PCR was used to isolate the B₂ esterase cDNA, since sequence was already known for the B₁ esterase gene and a B esterase anti-serum was not available, thereby preventing the screening of an expression library.

2.1 INTRODUCTION

The major mechanism of OP resistance in *Culex* mosquitoes is the elevated activity of one or more carboxylesterases. The most common elevated esterase pattern found in OP resistant *Cx quinquefasciatus* populations is that of A₂ and B₂ esterases (Villani *et al.*, 1983; Raymond *et al.*, 1987; Hemingway *et al.*, 1990; Wirth *et al.*, 1990; Peiris & Hemingway, 1993; Rivet *et al.*, 1994) although elevated B₁ esterase is also found on more than one continent (Georghiou & Pasteur, 1978; Bisset *et al.*, 1990). The B₁ esterase gene from a Californian strain of *Cx quinquefasciatus*, TEM-R, resistant to the OP temephos, has been cloned and sequenced (Mouches *et al.*, 1990). The gene codes for a protein of 540 amino acids and a partial predicted amino acid sequence for a B₂ esterase (Mouches *et al.*, 1990) indicates that the two proteins (B₁ and B₂ esterase) have a high level of homology. The elevated B₁ esterase activity in the TEM-R strain has been shown to be due to amplification of the B₁ esterase gene (Mouches *et al.*, 1986). The gene sits within an amplicon of approximately 25 kb and up to 250 copies are

found in the TEM-R genome, constituting about a 2% increase in genome size (Mouches *et al.*, 1990).

To determine the B₂ esterase cDNA sequence and the underlying mechanism of B₂ esterase elevation in the mosquito *Cx quinquefasciatus*, the PelRR strain was used. The 'Pel' colony was collected in 1984 from Peliyagoda in Sri Lanka. It was heterogeneous for OP resistance but the major resistance mechanism was increased activity of the carboxylesterases A₂ and B₂ (Peiris & Hemingway, 1990a; 1990b).

Oligonucleotide primers were devised based on the homology of the B₁ esterase gene sequence with that of acetylcholinesterase from *An. stephensi* (Hall & Malcolm, 1991). The primers were used to amplify an internal region of putative cDNA coding for B₂ esterase from the PelRR strain. Using modified RACE (rapid amplification of cDNA ends), the whole B₂ esterase cDNA was isolated. The mechanism of B₂ esterase elevation was determined by studies on genomic DNA from the PelRR and PelSS strains. It has been suggested, on the basis of the kinetics of the purified proteins, that the A₂ and B₂ esterases from different *Cx quinquefasciatus* strains are different (Kettermann *et al.*, 1993; Karunaratne *et al.*, 1993c). Using internal oligonucleotide primers identical to nucleotide sequence in both the B₁ and B₂ esterase genes, B₂ esterase gene sequences were amplified by PCR from the genomic DNA of three more *Cx quinquefasciatus* strains with elevated A₂ and B₂ esterases. The strains Dar91 and Tanga85 were collected respectively from Dar es Salaam and Tanga in Tanzania and SPerm was collected from Jeddah, Saudi Arabia. The region amplified covered two introns (2 and 3) in the B₁ esterase gene (Mouches *et al.*, 1990). The degree of identity

between the B₂ gene sequences would show if different genes coding for the B₂ esterase existed.

2.2 MATERIALS AND METHODS

2.2.1 Mosquito strains

A heterozygous population (Pel) of *Cx quinquefasciatus* Say was collected from Peliyagoda in 1984. It was selected to give a susceptible strain, PelSS and a resistant strain, PelRR (Peiris & Hemingway, 1990a; Amin & Peiris, 1990). PelRR was 31-fold more resistant to the OP temephos than PelSS and also showed broad spectrum cross-resistance to a range of OPs (Peiris & Hemingway, 1990b). Classical genetics indicated that the resistance in this strain was not consistent with a single major gene, although the resistance is entirely esterase-based (Peiris & Hemingway, 1990b; 1993).

The PelSS strain was selected by single family selection from the Pel strain as described by Amin & Peiris (1990).

The PelRR strain was selected from the same Pel parental strain by mass selection with temephos as described by Peiris & Hemingway (1990a). Since then insecticide resistance in this strain has been maintained by exposing fourth instar larvae of every third generation to the LD₅₀ concentration of temephos.

The Tanzanian strains, Dar91 and Tanga85 of *Cx quinquefasciatus*, were collected from Dar es Salaam and Tanga respectively. The Dar91 strain originated from a resistant field population which had been selected with chlorpyrifos and then with fenitrothion since 1988. The strain was colonised in 1991. Since colonization the

strain was maintained without selection pressure. The Tanga85 strain originated from a chlorpyrifos resistant field population and was colonized in 1985. The strain has been maintained with intermittent chlorpyrifos selection pressure. The field sites for Dar91 and Tanga85 are approximately 200 km apart. SPerm was collected from Jeddah, Saudi Arabia in 1989. Field selection of this population had been with temephos and then a wide range of pyrethroids. Immediately after colonization, it was selected for 20 generations with permethrin and subsequently selected intermittently with malathion and temephos.

2.2.2 Isolation of total RNA

The method used was an adaptation of Sambrook *et al.*, (1989). About 1 g of wet weight fourth instar larvae were thoroughly ground under liquid nitrogen. The larval homogenate was added to 20 volumes of guanidinium thiocyanate buffer [4.0 M guanidinium thiocyanate, 0.1 M Tris-HCl (pH 7.5), 1% (v/v) β -mercaptoethanol, 0.5% (w/v) sodium lauryl sarcosinate]. After vortexing, the homogenate was centrifuged at 5000g for 20 minutes. The supernatant was loaded onto a cushion of DEPC treated 5.7 M CsCl, 0.01 M EDTA (pH 7.5). After centrifugation at 20°C for 20 hours at 40000 rpm, the sedimented RNA pellet was removed and washed with 70% (v/v) ethanol and resuspended in 500 μ l TET [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)]. After phenol-chloroform and chloroform extraction the RNA was precipitated with the addition of 0.1 volumes of DEPC treated 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold ethanol. The pellet was collected by centrifugation at 12000g for 10 minutes and after being washed with 70% (v/v) ethanol was resuspended in a small volume of formamide and stored at -70°C. To precipitate the RNA, 3 volumes of ethanol were added.

2.2.3 Isolation of mRNA from total RNA

Messenger RNA was isolated using the PolyATract mRNA Isolation System IV (Promega). In this system, a biotinylated oligo(dT) primer base paired with the polyA(+) region of the mRNA. The mRNA was then captured with MagneSphere streptavidin paramagnetic particles which bind the biotinylated oligo(dT). After washing at high stringency to remove the polyA(-) RNA, the mRNA was eluted with ribonuclease-free deionized water and stored at -70°C. The whole procedure was performed in a single tube and this greatly decreases the chance of contaminating the RNA with ribonucleases.

2.2.4 cDNA synthesis from mRNA

Copy DNA was synthesised with the Riboclone cDNA Synthesis System (Promega) using an oligo dT adaptor primer [5' GACTCGAGTCGACATCGA-(dT₁₇) 3']. In a microcentrifuge tube, the primer and 2 µg of mRNA were added in a total volume of 15 µl water. After heating to 70°C for 5 minutes the tube was cooled to room temperature to allow the annealing of the primer to the mRNA. First strand synthesis of cDNA was carried out in a final volume of 25 µl at 42°C for one hour in the first strand buffer [50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl₂, 0.5 mM spermidine, 10 mM DTT, 4 mM sodium pyrophosphate, 1 mM dNTPs, 30 units rRNasin ribonuclease inhibitor (Promega), 30 units AMV reverse transcriptase (Promega)]. The AMV reverse transcriptase synthesises first strand cDNA from the mRNA template. Second strand cDNA synthesis was carried out in the same tube as that for first strand synthesis. Additional components were added to the tube to a final volume of 120 µl. Synthesis was carried out at 14°C for 4 hours in second strand buffer [50 mM Tris-HCl (pH 7.6), 100 mM KCl, 5 mM MgCl₂, 50 µg/ml bovine serum albumin, 5 mM DTT, 0.8

units RNase H (Promega), 23 units DNA polymerase I (Promega), 0.2 mM dNTPs, 0.1 mM spermidine, 0.8 mM sodium pyrophosphate]. RNase H creates nicks and gaps in the hybridized mRNA strand, which provides 3'-OH priming sites for DNA synthesis and repair by DNA polymerase I. The cDNA was extracted with phenol/chloroform and precipitated with 0.3 M sodium acetate and 2.5 volumes of cold (-20°C) ethanol. The cDNA was washed with 70% ethanol, dissolved in TET and stored at -20°C.

2.2.5 Isolation of B esterase cDNAs

2.2.5.1 PCR

Primers were used to isolate an internal fragment of the B esterase cDNA by PCR. Primers were constructed based on the knowledge of the B₁ esterase sequence (Mouches *et al.*, 1990) and of the *An. stephensi* acetylcholinesterase sequence (Hall & Malcolm, 1991). The primer set 5' T/C-T-A/G-AC-C/A/G-GT-G/C/T-C-AGAC 3' and 5' G/A-CA-G/A-TT-T/A/G/C-GG-A/G-TC-G/A/T/C-CC 3' were used to amplify a product of 1440 base pairs. The 50 µl PCR reaction contained 5 ng of double stranded cDNA, 50 ng of each primer, 0.5 mM dNTPs, 2 mM MgCl₂ and was buffered in *Taq* DNA polymerase buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100]. The reaction mixture was overlaid with mineral oil and heated in a DNA Thermal Cycler (Hybaid) to 94°C for 3 minutes. The mixture was removed and 2.5 units of *Taq* DNA polymerase (Promega) were added to the reaction which was then heated to 94°C for a further 3 minutes. Thirty five cycles of amplification were then carried out using a step programme (37°C, 3 minutes; 72°C, 3 minutes and 94°C, 3 minutes).

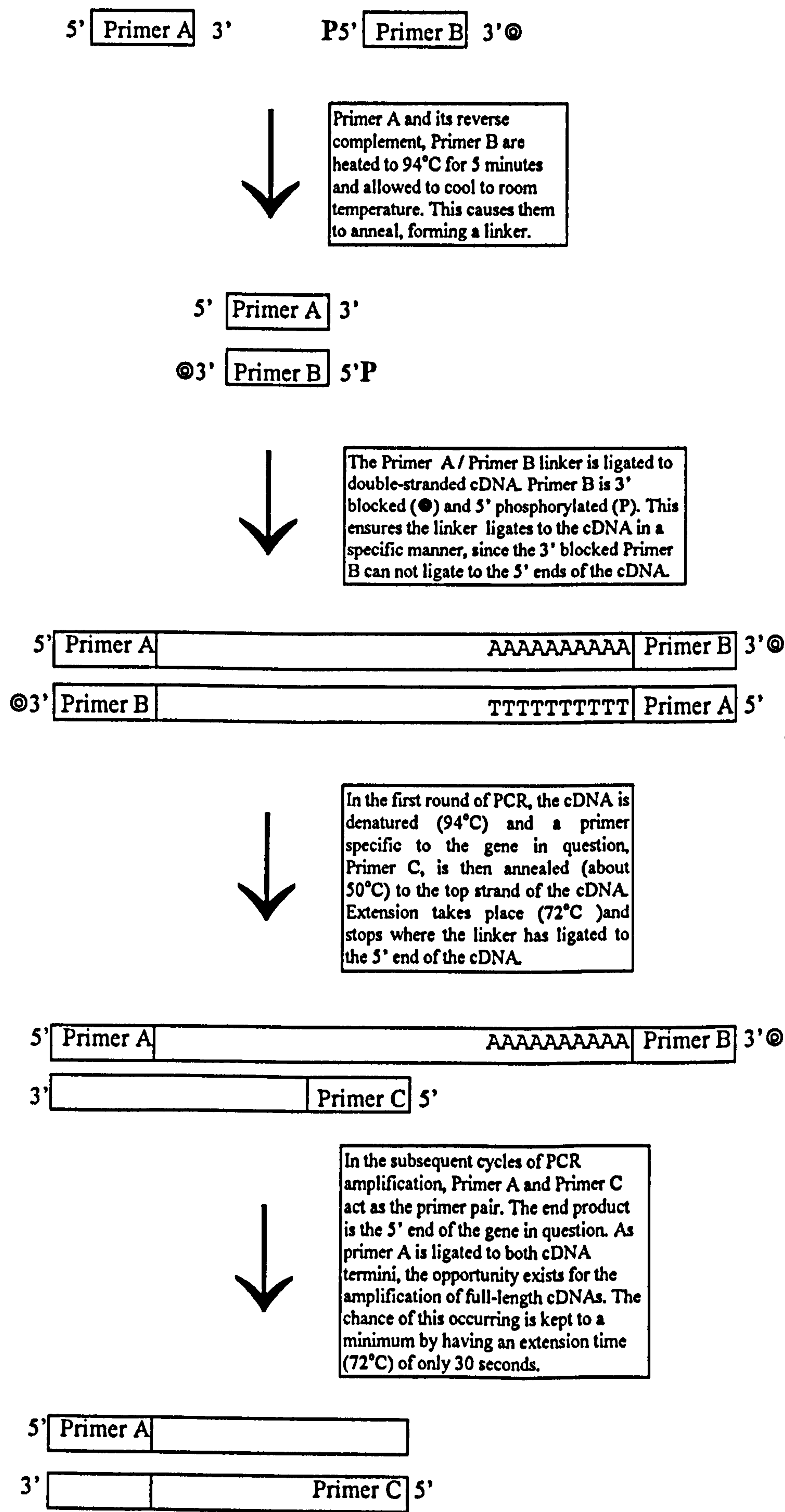
2.2.5.2 *Rapid amplification of 3' cDNA ends (3' RACE)*

The 3' B esterase cDNA was amplified using an internal forward primer and the adaptor primer sequence (5' GACTCGAGTCGACATCGA 3'; METHODS 2.4) for the reverse primer. FIGURE 2.1 shows how the 3' RACE was used to amplify the 3' end of the carboxylesterase cDNA. Briefly, an internal esterase primer was annealed to the reverse strand of the cDNA and extension with *Taq* DNA polymerase (Promega) lead to the synthesis of the 3' end of the cDNA. The adaptor primer, which annealed to the very end of the forward strand of the cDNA was then used in conjunction with the esterase specific primer to amplify the 3' end of the gene in a PCR. The PCR was carried out with first strand cDNA and the PCR conditions were the same except for the addition of 40 ng of first strand cDNA, an increased annealing temperature of 50°C and an extension time in the first cycle of 4 minutes.

2.2.5.3 *The modified rapid amplification of 5' cDNA ends (5' RACE)*

The 5' B esterase cDNAs could not be amplified by standard RACE techniques. 5' RACE uses the addition of a linker at the 5' end of the first strand cDNA pool with RNA ligase (Tessier *et al.*, 1986; Troutt *et al.*, 1992). The addition of this linker is difficult as the reaction with the RNA ligase does not favour the linker addition. To overcome this, a double stranded linker was added to blunted double stranded cDNA. The 50 µl blunting reaction contained 1 µg of cDNA, 0.5 mM dNTPs and was buffered in T4 DNA polymerase repair buffer [18 mM (NH₄)₂SO₄, 66 mM Tris-HCl (pH 8.3), 6.6 mM MgCl₂ and 10 mM β-mercaptoethanol]. Two units of T4 DNA polymerase were added and the reaction was incubated for 15 minutes at 37°C. After phenol-chloroform extraction the cDNA was precipitated, resuspended and ligated to the double stranded linker.

FIGURE 2.1 The rapid amplification of 3' cDNA ends (3' RACE).

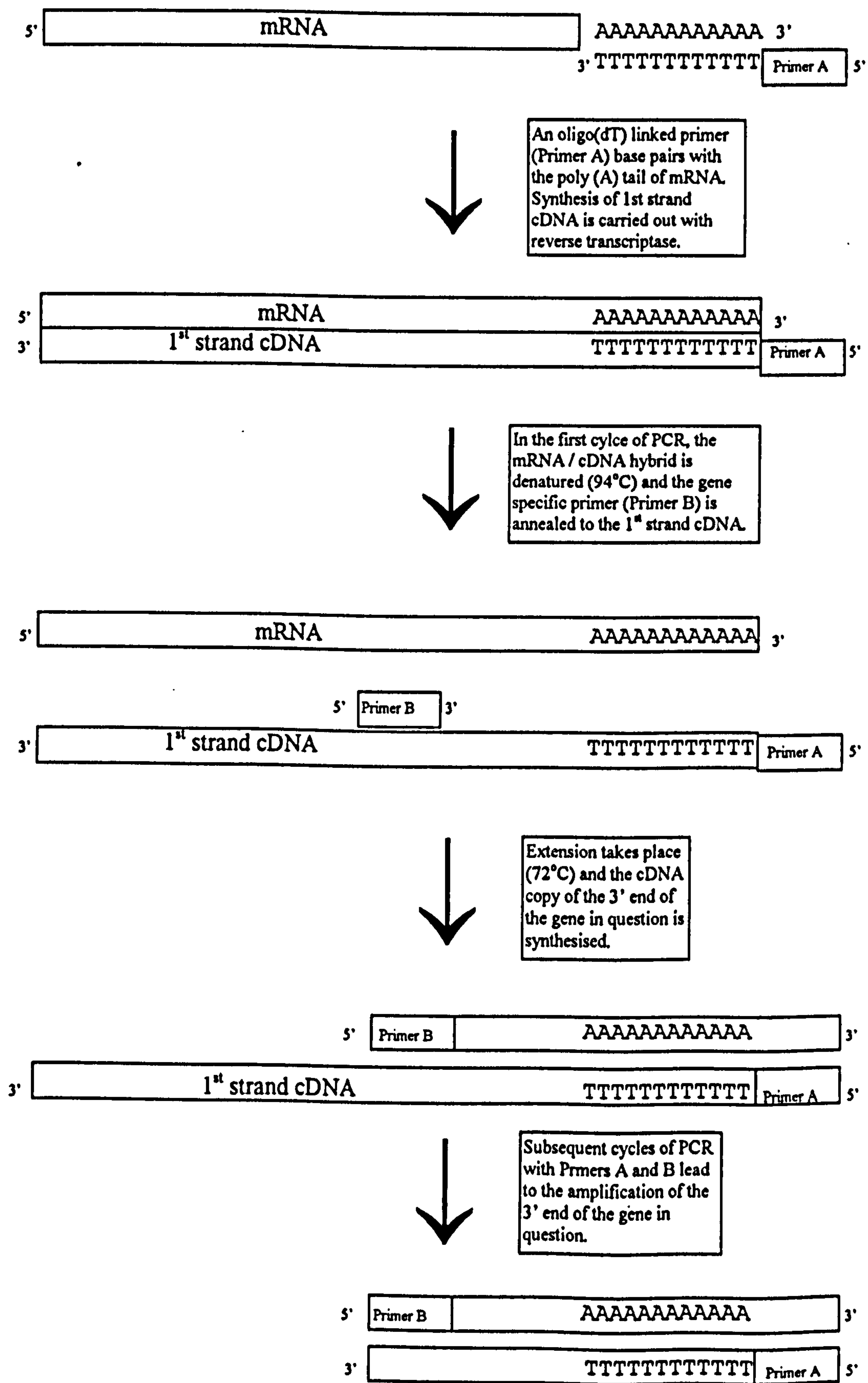


The linker used was made up of two oligonucleotides, 5' TGACCGGCAGCAAAATG 3' (Primer A) and its reverse complement 5' CATTTTGCTGCCGGTCA 3' (Primer B). Primer B was 5' phosphorylated with T4 polynucleotide kinase (Stratagene) and 3' blocked with the addition of ddATP using terminal deoxynucleotidyl transferase (Stratagene), following the manufacturers recommendations. This allowed the direction specific ligation of the linker to the cDNA pool, because only the 5' phosphorylated Primer B was able to ligate to the 3' hydroxyl group of the cDNA. Primer B was also 3' blocked to prevent its ligation to the cDNA. **FIGURE 2.2** shows how the 5' RACE was used to amplify the 5' end of an esterase specific cDNA. Equimolar amounts of the two oligonucleotides were heated to 94°C for 2 minutes and annealed by cooling to room temperature. A 100-fold molar excess of linker was ligated to the cDNA at 14°C for 16 hours with T4 DNA ligase (Promega). The 5' B esterase cDNA was amplified using an internal reverse primer, which bound to the forward strand of the cDNA and Primer A, which was linked to the 5' end of the cDNA. Forty nanograms of cDNA were used directly from the ligation reaction for the PCR. The PCR conditions were the same as **METHODS 2.2.5.1**, apart from an increased annealing temperature of 50°C and decreased cycling times of 1 minute for each step.

2.2.5.4 Subcloning into the pBluescript thymidine (T)-vector

The amplified B esterase cDNA products from the PCR reactions were subcloned into pBluescript (Stratagene). The vector was digested with *EcoRV* and single thymidines were added to the blunt ends of the linearised vector (Marchuk *et al.*, 1991) to, creating T overhangs. This facilitates the ligation of the PCR product into the T-vector since *Taq* DNA polymerase incorporates complementary

FIGURE 2.2 The modified rapid amplification of 5' cDNA ends (5' RACE).



adenine (A) overhangs into PCR products and these base pair with the thymidines of the vector.

2.2.6 Isolation of genomic DNA

The method used was an adaptation of Miller *et al.*, (1988). About 1 g of wet weight fourth instar larvae were ground under liquid nitrogen. The homogenate was added to 10 volumes of extraction buffer [10 mM Tris-HCl (pH 8), 0.1 M EDTA (pH 8), 0.5% (w/v) SDS, 20 μ g/ml pancreatic RNase]. After incubation for an hour at 37°C proteinase K was added to a final concentration of 100 μ g/ml and the homogenate was incubated at 50°C for three hours. After cooling on ice for 10 minutes, 0.35 volumes of saturated NaCl were added to precipitate protein. The homogenate was well mixed and stored on ice for a further 5 minutes, then centrifuged at 16000g for 20 minutes. The supernatant was removed and the DNA precipitated by adding an equal volume of isopropanol. The DNA was resuspended in 7.5 ml of TET containing 20 μ g/ml RNase, incubated at 37°C for an hour and then extracted with phenol, phenol/chloroform and finally chloroform. After precipitation with ethanol, the DNA was resuspended in a small volume of TET and stored at 4°C.

2.2.7 Genomic DNA studies

A PelRR B₂ esterase cDNA fragment was used as a probe to determine the haplotype of the B₂ esterase. 10 μ g of genomic DNA was digested to completion with *Eco*RI and separated by gel electrophoresis through 0.8% (w/v) agarose. The DNA was transferred to nylon membranes (Amersham) and hybridised with the ³²P-labelled probe (specific activity > 2 x 10⁶ cpm/ μ g) at 65°C for 16 hours in hybridisation buffer [5 x Denhardt's reagent (50 x Denhardt's reagent contains 5

g of Type 400 Ficoll, 5 g of polyvinylpyrrolidone, 5 g of Fraction V BSA and water to 500 ml), 6 x SSC (20 x SSC contains 175.3 g of NaCl and 88.2 g of sodium citrate in 1 litre of water, adjusted to pH 7.0 with NaOH), 0.1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate, 5% (w/v) PEG 8000 (polyethylene glycol with an average molecular weight of 8000) and 100 µg/ml boiled, sheared herring sperm DNA]. The final washes were at 65°C in 0.1 x SSC and 0.1% (w/v) SDS for 20 minutes. The blot was exposed to autoradiographic film overnight at -70°C with intensifying screens.

2.2.8 Isolation of genomic DNA sequences of B esterases

2.2.8.1 PCR

Genomic DNA coding for the B₂ esterases from several resistant strains and the susceptible strain, PelSS, were amplified using *Pfu* DNA polymerase (Stratagene) which has a 3' to 5' proofreading activity. This is associated with a 12-fold increase in fidelity of DNA synthesis over *Taq* DNA polymerase. Primers that were used initially for sequencing the PelRR B₂ cDNA were used to PCR amplify genomic DNA. It was assumed that the B₂ esterase gene sequence would be similar to that of the B₁ esterase gene and the amplified DNA fragment covered introns 2 and 3 of the B₁ esterase gene. Each 50 µl reaction contained 100 ng of genomic DNA, 50 ng of each primer, 1.25 units of *Pfu* DNA polymerase and was performed in reaction buffer [200 mM Tris-HCl (pH 8.75), 100 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgCl₂, 1% Triton X-100, 1 mg/ml BSA]. All the components, apart from the polymerase were mixed in a 0.5 ml microcentrifuge tube and overlaid with mineral oil. The *Pfu* was added to the mixture when the first cycle had reached 94°C for 3 minutes. The first cycle of the PCR was 94°C, 7 minutes; 47°C, 3 minutes; 72°C, 10 minutes. The subsequent 35 cycles were 94°C, 2

minutes; 47°C, 2 minutes and 72°C, 4 minutes.

2.2.8.2 Subcloning

The products of a PCR reaction with *Pfu* DNA polymerase do not have adenine overhangs and are blunt ended. They were subcloned into the vector pCRScript (Stratagene). This vector, when ligated to itself, forms the rare restriction site *SfiI*. However, the presence of an insert destroys this site. Therefore, the ligation is carried out in the presence of T4 DNA ligase and *SfiI* and this leads to the majority of ligated products containing an insert.

2.2.9 DNA sequencing

DNA inserts from purified plasmid DNA were sequenced with Sequenase Version 2.0 (USB Biochemical Corporation) using the manufacturers recommendations or with the ALF Automatic Sequencer (Pharmacia). In the case of cDNA and genomic DNA products isolated by PCR, at least two separate PCR clones were sequenced. Sequencing of both strands of the B esterase clones was completed using primers complementary to pBluescript and a large number of primers complementary to the B esterase nucleotide sequence. For each B esterase cDNA, overlapping PCR products were sequenced to ensure isolation of a single cDNA sequence. Alignments of the nucleotide sequences and the inferred amino acid sequences were carried out using the LASERGENE package (DNASTAR LTD.).

2.3 RESULTS

The novel approach used for the amplification of the 5' end of the cDNAs relied upon the ligation of a double stranded linker to the double stranded cDNA pool.

Oligo A of the linker was 5' phosphorylated in order to allow its ligation to the cDNA pool. The linker will also ligate to the 3' end of the cDNA pool, which could lead to the amplification of the whole cDNA pool in the PCR (as Oligo A could anneal to both ends of the cDNA). However, the specificity of the internal primer and the short extension time of the PCR cycle kept the amplification of non-specific sequences to a minimum.

As was seen in the TEM-R B₁ esterase gene (Mouches *et al.*, 1990), the PelRR B₂ esterase cDNA had a short region of 5' non-coding sequence. This was followed by an open reading frame of 1623 base pairs starting with an initial ATG codon and ending with a TGA stop codon. There followed, a short 3' non-coding sequence and a polyA(+) tail. The open reading frame of the PelRR B₂ esterase cDNA coded for a protein of 540 amino acids, as for the TEM-R B₁ esterase. **FIGURE 2.3** shows the nucleotide sequence and the inferred amino acid sequence of the full length PelRR B₂ esterase cDNA.

Nucleotide changes between gene sequences can lead to changes in the amino acid sequence but many are silent, as often a mutation of the nucleotide at the third base of the codon has no effect on the translated amino acid sequence. The ratio of silent to non-silent changes can be used to determine whether a sequence is under active positive selection pressure. The 54 silent nucleotide differences between the open reading frames of PelRR B₂ and TEM-R B₁ esterase account for 77.1% of the 70 nucleotide changes. This comparison is shown in **FIGURE 2.4**. There are 16 amino acid differences between the two B esterases and the identity between the two esterase open reading frames is 95.7% at the nucleotide level and 97.0% at the amino acid level. The partial amino acid sequence for a B₂

FIGURE 2.3 continued

310v 320v 330v 340v 350v 360v 370v 380v 390v 400v
CGGTGATGCTGTACATTTACGGCGGTGGCTTACGGAAGAACCGAACCAGCGGATTCGTACGGTCCGGATTCTTGGTTCAGAAGGATATCGTTTGGT
P V M L Y I Y G G G F T E G T S G T E L Y G P D F L V Q K D I V L V 130^
110^

410v 420v 430v 440v 450v 460v 470v 480v 490v 500v
GTCGTTCAACTACCGCATTTGGGGCGTTGGGATTCTGTGTTGTCAATCGGAGCAGGATGGCGTACCCGGTAATGCCGGAATCAGAACTTGGCC
S F N Y R I G A L G F L C C Q S E Q D G V P G N A G L K D Q N L A 160^
140^ 150^

510v 520v 530v 540v 550v 560v 570v 580v 590v 600v
ATTCGGTGGGTTCTGGAGAACATTGCCCGCCTTTGGAGGGACCCGAGCGGTGACCCCTGGTCGGTCATAGCGCAGGTGCCGCTTCGGTCAGTATCATC
I R W V L E N I A A F G G D P K R V T L V G H S A G A S V Q Y H 200^
170^ 180^ 190^

610v 620v 630v 640v 650v 660v 670v 680v 690v 700v
TGATCTCGGATGCTTCCAAGGACTTGTTCAGCGGGCTATCGTAATGTCTGGAGTACGTATAACAGTTGGTCGTTGACCCAGGCAACGCAACTGGGTTGA
L I S D A S K D L F Q R A I V M S G S T Y N S W S L T R Q R N W V E 230^
210^ 220^

710v 720v 730v 740v 750v 760v 770v 780v 790v 800v
GAAGTTGGCGAAGGCCATCGGTTGGGATGGACAGGGTGGTGAGTCCGGTGCGTTGAGATTCTTGAAGCTGCCAAACCCGGAGGACATTGTTGCTAACCCAG
K L A K A I G W D G Q G G E S G A L R F L K A A K P E D I V A N Q 260^
240^ 250^

810v 820v 830v 840v 850v 860v 870v 880v 890v 900v
GAGAAGCTTCTGACTGACCAGGACATGCAGGACGATATCTTTACTCCGTTTGGACCTACCGTTGAACCGTACCTGACGGAACAGTGCAATGATCCGAAGG
E K L L T D Q D M Q D I F T P F G P T V E P Y L T E Q C M I P K 300^
270^ 280^ 290^

910v 920v 930v 940v 950v 960v 970v 980v 990v 1000v
AACCGTTTGAGATGGCTCGAACTGCTTGGGGTGACAAAGATAGATATCATGATCGGAGGAACCTTCTGAGGAAGGACTGCTGCTGCTGCAAAAGATCAAGTT
E P F E M A R T A W G D K I D I M I G G T S E E G L L L Q K I K L 330^
310^ 320^

1010v 1020v 1030v 1040v 1050v 1060v 1070v 1080v 1090v 1100v
GCAACCGGAACACTGTCCCATCCTCATCTGTTCCTGGGAATGTTCCTCCAAACTTGAAGATCAGCATGGAAAAACGAATCGAGTTTGTGCTGCCAACTG
Q P E L L S H P H L F L G N V P P N L K I S M E K R I E F A A K L 360^
340^ 350^

FIGURE 2.3 continued

1110v 1120v 1130v 1140v 1150v 1160v 1170v 1180v 1190v 1200v
AAGCAACGTTACTACCCGATAGCAGTCCTTCGATGGAGAACAACCTGGGATACGTTTCATATGATGTCCGACCGGGTCTTCTGTGCACGGCCTGCACCGCA
K Q R Y Y P D S S P S M E N L G Y V H M M S D R V F W H G L H R
370^ 380^ 390^ 400^

1210v 1220v 1230v 1240v 1250v 1260v 1270v 1280v 1290v 1300v
CCATCCTCGCCGAGCGGCTCGATCGCGCGCCGACCTTCGTGTACCGGATCTGTCTGGAGTTCTTACAACCACTACCGCATCATGATCGA
T I L A R A A R S R A R T F V Y R I C L D S E F Y N H Y R I M I D
410^ 420^ 430^

1310v 1320v 1330v 1340v 1350v 1360v 1370v 1380v 1390v 1400v
CCCGAAGCTGCGGCGCACGGCCCATGCGGACGAGCTGTCTTCCAACTTCAACCCAGCAGGTCCCCGGCAAGGAAACGTTTCGAGTACCGCGGT
P K L R G T A H A D E L S Y L F S N F T Q Q V P G K E T F E Y R G
440^ 450^ 460^

1410v 1420v 1430v 1440v 1450v 1460v 1470v 1480v 1490v 1500v
CTGCAAAACGCTGGTCGATGTGTTCACCGCGTTCGTCATCAACGGGGATCCAAACTGTGGCATGACGGCGGAGAGCGGTGTGTTCGAGCCGAAACGCGC
L Q T L V D V F T A F V I N G D P N C G M T A K S G V V F E P N A
470^ 480^ 490^ 500^

1510v 1520v 1530v 1540v 1550v 1560v 1570v 1580v 1590v 1600v
AGACGAAGCCACGTTCAAGTGTCTGAACATTCGCAACGACGGGGTGCGGTTTCGTTGATTATCCGGGATGCGGACCGCATTTGGACATGTGGGACGCAATGTA
Q T K P T F K C L N I A N D G V A F V D Y P D A D R L D M W D A M Y
510^ 520^ 530^

1610v 1620v 1630v 1640v 1650v 1660v 1670v 1680v
CGTGAACGATGAGCTGTTTTGAGGGGACAGTTTTAAATATCCCTGTTGAGATTGTGTAGGTTAAATACACTATTTATCAGT\$
V N D E L F X
540^

esterase has previously been published (Mouches *et al.*, 1990) and the three amino acid sequences are compared in FIGURE 2.5. There are three amino acid differences between the PelRR B₂ and partial B₂ esterase amino acid sequences.

It has yet to be demonstrated whether the B esterases are glycosylated. There are 3 possible sites of N-linked glycosylation (this can occur at Asn-Xxx-Ser or at Asn-Xxx-Thr in an amino acid sequence where Xxx is not Pro) in the three B esterases and these are shown in FIGURE 2.5. The three glycosylation sites do not occur in all the amino acid sequences.

To date, it has been shown that the B₁ esterase haplotype has an amplified 2.1 kb *EcoRI* genomic DNA fragment whilst the B₂ esterase haplotype has an amplified 9 kb *EcoRI* fragment (Raymond *et al.*, 1991). To determine if the PelRR B₂ esterase haplotype conformed to this pattern, a 1350 bp PelRR B₂ esterase cDNA fragment was hybridised to *EcoRI* digested genomic DNA from PelRR. The resulting pattern is shown in FIGURE 2.6. The amplified (when compared to the PelSS *EcoRI* fragment) *EcoRI* fragment from the PelRR strain was 9 kb. There was also a low intensity fragment at 3.3 kb in PelRR which was of a similar size and intensity to the fragment in PelSS.

The partial genomic B₂ esterase gene sequence from the four *Cx quinquefasciatus* strains, PelRR, Tanga85, SPerm and Dar91 were all exactly the same, throughout both the exon and intron sequences. The enzyme *Pfu* polymerase (Stratagene) was used for the DNA isolation as it has a proof reading capability and thus was less likely to introduce mistakes. The region sequenced covered two introns which were in identical positions as introns 2 and 3 of the B₁ esterase gene. The 1061

FIGURE 2.5 The identities between the inferred amino acid sequences of the B esterases from three *Culex quinquefasciatus* strains. The strains used were PelRR (esterase B₂), TEM-R (esterase B₁, Mouches *et al.*, 1990) and a previously published amplified B₂ esterase (Raymond *et al.*, 1991). The amino acid sequence for PelRR esterase B₂ is shown in the top row. The same amino acid in the other esterases is shown by an asterisk. The previously published esterase B₂ sequence starts at position 269 and ends at position 483. Lower case letters are used when one sequence differs from any other sequence and X represents the stop codon. Possible N-linked glycosylation sequences are underlined. The three amino acids (S¹⁹¹, E³²⁴ and H⁴⁴²) which are thought to make up the active site triad, based on an alignment of a number of carboxylesterases and related sequences (Cygler *et al.*, 1993) are in bold type.

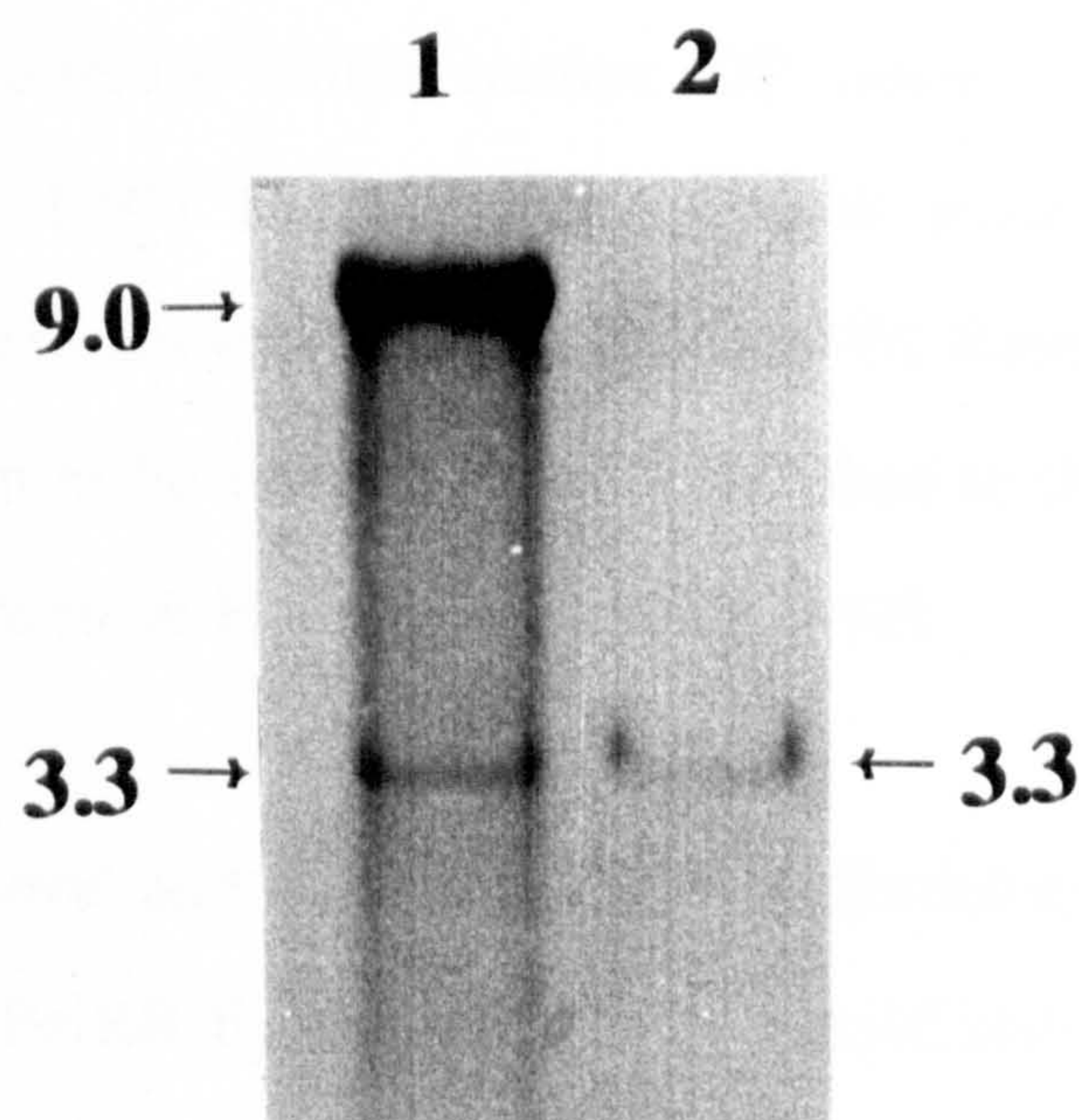
	10v	20v	30v	40v	50v	60v	70v	80v	90v	100v
PelRR	MSLESLTVQTKYGPVVRGKR	sVSLLGQEYVSFQGI	PYARAPEGELR	FKAPVPPQn	WTETLDCs	QQCEPCYHF	DRRLQKIVGCEDSLKIN	VF	AKEIN	PSkPLP
TEM-R	*****	***** <u>η</u>	*****	*****	*****	*****	*****	*****	*****	*****t
	110v	120v	130v	140v	150v	160v	170v	180v	190v	200v
PelRR	VMLYIYGGGFTSGTE	LGTPDFLVQKD	IVLVSFNYRIGAL	FLCCQSEQDGV	PGNAGLKDQNL	AI	RWVLENI	AAFGGDPKRV	TLv	GH
TEM-R	*****	*****	*****	*****	*****	*****	*****	*****	*****a	*****
	210v	220v	230v	240v	250v	260v	270v	280v	290v	300v
PelRR	SDASKDLFQRAIV	MSGSTYn	SWSLTRQRN	WVEKLAKAIG	WDGQGESGAL	RFLkAAKPEDIV	AnQEKL	LLTDQDMQDD	IF	TFPGPTVEPYL
TEM-R	*****	*****g	*****	*****	*****	*****r	*****h	*****	*****	*****i
B2	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****e
	310v	320v	330v	340v	350v	360v	370v	380v	390v	400v
PelRR	EMARTAWGDKID	IMIGTSEEG	LLLLQKIKLq	PELLSHPHL	FLGNVPPNL	KISM	EkRIEFAAKL	KQRYYPDS	sPSMEN	NLGyVHMMSDRVFWHGLHRTILA
TEM-R	*****	*****	*****h	*****	*****	*****	*****i	*****	*****	*****
B2	*****r	*****	*****h	*****	*****	*****	*****g	*****	*****	*****

FIGURE 2.5 continued

	410v	420v	430v	440v	450v	460v	470v	480v	490v	500v
Pe1RR	RaARSRARTFVYR	ICLDSEFFYNHYR	IMMIDPKLRGTA	HADEL	SYLFSNFTQQV	PGKETFEYRGLQ	TLVDVftAFVING	DPNCGMTAKs	GVVFE	PNAQTKPT
TEM-R	*a*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
B2	*g*****	*****	*****	*****	*****	*****	*****	*****	*****	*****

	510v	520v	530v	540v
Pe1RR	FKCLNIANDGVA	FVDYPDADRLD	MDAMYVNDEL	FX
TEM-R	*****	*****	*****	*****

FIGURE 2.6 Southern blot and hybridisation of PelRR and PelSS digested genomic DNA. Genomic DNA from the *Culex quinquefasciatus* strains, PelRR (OP resistant; **Lane 1**) and PelSS (susceptible; **Lane 2**), was digested with *Eco*RI, separated on a 0.8% agarose gel, blotted and hybridised with a 1350 bp PelRR B₂ esterase cDNA probe. The final wash was in 0.1 x SSC and 0.1% (v/v) SDS at 60°C for 20 minutes. The blot was exposed to autoradiographic film overnight at -70°C with intensifying screens. The size of the fragments are in kilobase pairs (kb).



bp partial genomic sequence of the B₂ esterase genes were compared with that of esterase B₁ (FIGURE 2.4). The PelRR open reading frame genomic and cDNA B₂ esterase sequences were exactly the same. This provides proof that the correct B₂ esterase gene had been cloned.

2.4 CONCLUSIONS

The Californian TEM-R resistant strain of *Cx quinquefasciatus* has an over-expressed B₁ esterase which confers OP resistance (Mouches *et al.*, 1986; Raymond *et al.*, 1989). The B₁ esterase genomic sequence and its inferred amino acid sequence are known (Mouches *et al.*, 1990). Resistance in the PelRR strain has been shown to be esterase based and linked to the elevation of the A₂ and B₂ esterases (Peiris & Hemingway, 1990a; 1993).

In this study, novel and established PCR methodology has been used to isolate the full-length PelRR B₂ esterase cDNA. Amplification of the B₂ esterase gene has previously been shown in resistant *Cx quinquefasciatus* populations (Raymond *et al.*, 1991). It was assumed that the B₂ esterase gene from the PelRR strain would also be amplified. This assumption appeared justified as Southern blot analysis shows that the B₂ esterase gene from the insecticide resistant PelRR strain is amplified when compared to that of the B esterase gene from the insecticide susceptible strain, PelSS. There was also a fragment of low intensity in the PelRR strain and a fragment of similar intensity was seen in hybridised genomic DNA from the susceptible PelSS strain. It seems likely that the susceptible strain, PelSS, contains a single copy of the B esterase gene. However, this has not been proved as yet. One way of determining the number of B esterase gene copies present in the PelSS strain would be to digest 10 µg of PelSS

genomic DNA with a restriction enzyme, and then separate the fragments by agarose gel electrophoresis. A B esterase DNA probe of known size would also be run out on the gel in a dilution series. Once the gel had been Southern blotted and hybridised with the same B esterase DNA probe, the signal for the PelSS could be equated to an exact amount of DNA. Knowing that the genome size of *Cx quinquefasciatus* is approximately 10^8 bp, calculations could be done to predict the number of copies of the gene that were present in the genome. The use of PCR to amplify the resistance linked B₂ esterase cDNA could also amplify the susceptible-linked allele(s) seen in the Southern blot. However, the resistance-linked B₂ esterase cDNA (and the related PCR product) would make up the vast majority of the total B esterase cDNAs present from the B esterase PCR amplification of PelRR cDNA assuming that the B₂ esterase gene was amplified and this in turn lead to the increased production of B₂ esterase mRNA. To ensure that the same B esterase cDNA was isolated, multiple overlapping PCR products were sequenced. The cDNAs were all identical and it was concluded that the resistance linked cDNA had probably been isolated.

The B₁ and B₂ esterases are both proteins of 540 amino acids. The high identities at the molecular level (95.7% at the nucleotide level and 97.0% at the amino acid level) between the TEM-R B₁ and PelRR B₂ esterases suggest that the genes are alleles. The majority (77.1%, 54) of the nucleotide differences between the PelRR B₂ and TEM-R B₁ esterase cDNAs do not lead to a change in their amino acid sequences. Nucleotide point mutations that do not lead to an amino acid change are phenotypically silent and this explains the large number present between the two genes. However, for amino acid changes to be tolerated, they must allow the function of the enzyme to be maintained. This is undoubtedly why the majority

of the nucleotide substitutions do not lead to amino acid changes. There are, however, 16 amino acid differences between the two proteins. The differences between the two esterase genes, based on nucleotide and Southern blot analysis of *EcoRI* digested genomic DNA, suggest that they arose from different amplification events. The TEM-R *EcoRI* fragment for esterase B₁ is 2.1 kb whereas PelRR has a B₂ esterase *EcoRI* fragment of 9 kb.

The 9 kb *EcoRI* fragment associated with the B₂ esterase from PelRR is the same as that reported for other B₂ genes (Raymond *et al.*, 1991). This is further evidence that the B₂ esterases arose from a single amplification event. A single 3.3 kb *EcoRI* fragment of low intensity was also seen in the PelRR and PelSS strains. This suggests that the PelSS strain carries a single B esterase allele and that the same allele could also present in the PelRR strain, although this alone does not confirm this. The B esterase gene associated with this fragment would have to be isolated from both PelRR and PelSS and sequenced to prove this. However, because the PelRR strain contains a highly amplified B₂ esterase gene, it would be difficult to isolate the under represented allele. The presence of a single *EcoRI* 9 kb amplified fragment suggests that the sequence around each B₂ esterase gene is similar. Further restriction enzyme digests would have to be carried out to try and deduce the size of the B₂ esterase gene amplicon. It is conceivable that the amplicons sit in a linear arrangement on a single chromosome. If this was the case, then a restriction enzyme that cut the amplicon only once would be needed to show this. Digesting genomic DNA with such an enzyme would give a single fragment on a Southern hybridisation if the amplicons were all the same distance apart. However, it has been suggested that the amplicon containing the TEM-R B₁ esterase has differing flanking sequences

(Mouches *et al.*, 1990) and this could lead to a number of fragments in enzyme digestion analysis, even if the amplicons were ordered in a linear fashion.

The B esterases have been purified from both PelRR and PelSS (Karunaratne *et al.*, 1993a; Karunaratne, 1994). The B₂ esterase from PelRR has a significantly higher affinity for the oxon analogues of the OPs than the B esterase from PelSS (Karunaratne, 1994). This implies that the two genes are coded for by different alleles and the different sized *Eco*RI fragments between the strains suggests this. Therefore, OP resistance in the PelRR strain is not only likely to be due to the amplification of a gene which codes for a B₂ esterase but also for an esterase which has a higher affinity for OPs than the esterase from the susceptible strain. It is not clear at present if the B₂ esterase gene was amplified after the introduction of OPs for the control of mosquitoes (in the last 40-50 years) or if the amplification occurred previously for other reasons. Carboxylesterases are found in mammalian liver where they are involved in the degradation of many xenobiotics. In the same way, mosquito carboxylesterases are likely to be involved in the degradation of xenobiotics and such compounds are in abundance in mosquito breeding sites. *Culex* mosquitoes favour polluted breeding sites and their larvae are opportunistic scavengers and thus come into contact with large numbers of xenobiotics. The mosquito would have come in to contact with many xenobiotics during its evolution and amplification of esterases involved in detoxication could have occurred during this time.

One possible N-linked glycosylation site is shared between the B₁ and B₂ esterases as well as each esterase having a unique site. However, since there is no amino-terminal leader sequence for either esterase, they are probably not exported from

the cell or glycosylated. This is also supported by the lack of binding of the B₂ esterases to ConA chromatography columns (Karunaratne, 1994).

Genomic sequence from four strains of *Cx quinquefasciatus* from Sri Lanka (PelRR), Tanzania (Tanga85, Dar91) and Saudi Arabia (SPerm) was exactly the same over 1061 base pairs. This included the homologues of introns 2 and 3 from the TEM-R B₁ gene. It is not clear yet if the B₂ esterase gene from PelRR has three introns (as the TEM-R B₁ gene does) but interestingly, the region of DNA surrounding the likely location of intron 1 from the PelRR B₂ could not be amplified by PCR. It is known that thermostable polymerases are inefficient at polymerising regions of high A and T content. Intron 1 of TEM-R B₁ is very long and has a high A and T content and if the B₂ gene were similar, this would explain the inability to amplify the intron from the four elevated B₂ esterase strains. The identity of the genomic sequence from the four strains suggests that they carry the same amplified gene. Differences in the kinetic characteristics of the B₂ esterases from these strains in their interaction with OPs have been reported (Kettermann *et al.*, 1993; Karunaratne *et al.*, 1993c; Karunaratne *et al.*, 1995). This data would seem to disagree with the sequence data. However assuming that the strains also carry other low copy number (probably only one) B esterase alleles not associated with resistance (as PelRR does), then these esterases could be co-purified with the amplified esterase and lead to the apparent differences in kinetics. It has been proposed that the amplified B₂ and B₁ esterase genes have spread around the world by the migration of resistant mosquitoes carrying the amplified genes (Raymond *et al.*, 1991). The evidence here supports the hypothesis for the B₂ amplification, as the sequences of the four B₂ esterase genes are identical. If the amplification of the B₂ esterase had

occurred independently in all four strains, it is likely that differences in the sequence of the amplified genes would be found.

It is difficult to explain the differences between the B₂ esterase amino acid sequence from PelRR B₂ and the previously partially sequenced B₂ esterase (Mouches *et al.*, 1990). The level of B₂ esterase amplification in the strain sequenced by Mouches *et al.* (1990) has not been reported. Knowing that resistant strains also carry low copy number (probably only one) alleles, it is possible that the strain had a low level of resistance (and therefore a low level of gene amplification) and that a low copy number (probably only one) allele has been partially sequenced by mistake. Sequencing errors can not be discounted either. In this study, however, genomic clones from four different *Cx quinquefasciatus* strains expressing A₂ and B₂ esterases were found to have exactly the same sequence, so it is unlikely that any sequencing errors have occurred in this study.

The two genomic sequences (PelRR B₂ and TEM-R B₁ esterase) were compared and showed high homology. Over the 1061 nucleotides, there were 80 differences and although the same introns in the B₁ and B₂ esterases genes occurred at the same positions, they were of different lengths. Within the introns, which covered a total of 118 nucleotides there were 27 differences, an identity of 77% compared to 94% identity between the exon sequences. Differences in length and sequence of introns clearly do not alter the amino acid sequence of a translated gene. However, the inter-strain termini of the B₁ and B₂ esterase gene introns are identical. This could be because the terminal sequences are vital for the efficient splicing of the introns in mRNA maturation.

CHAPTER 3

CHAPTER 3: ISOLATION OF TWO MORE *CULEX QUINQUEFASCIATUS* B ESTERASE CDNAS: B₁ FROM THE OP RESISTANT STRAIN, MRES AND 'SUSCEPTIBLE' B FROM THE OP SUSCEPTIBLE STRAIN, PELSS

This CHAPTER describes the isolation of a *Cx quinquefasciatus* B₁ esterase cDNA, a mRNA copy of a uniquely amplified B₁ esterase gene and the isolation of a B esterase cDNA from an *Cx quinquefasciatus* OP susceptible strain. As in CHAPTER 2, PCR was used, since the unavailability of a B esterase anti-serum, prevented the screening of expression libraries.

3.1 INTRODUCTION

It is clear from CHAPTER 2 that the PelRR B₂ and the TEM-R B₁ esterase genes are allelic. It has also been shown by genomic Southern blot analysis, that the PelSS OP susceptible strain, which was derived from the same parental strain as PelRR, contains a low copy number (probably only one) B esterase allele. The same allele seems also to be present in the PelRR strain along with a highly amplified B₂ esterase allele. The PelRR and PelSS B esterases differ in their ability to bind the oxons of the OPs (Karunaratne, 1994). The PelRR B₂ esterase has a greater affinity for the oxons than the B esterase from PelSS and this, in association with the amplification of the gene and subsequent increased levels of protein synthesis, leads to the observed resistance in the PelRR strain.

To investigate the differences between the B esterase alleles of the OP resistant and susceptible strains, a B esterase cDNA was isolated from the PelSS strain and compared with the previously isolated B₂ esterase from PelRR. A piece of

genomic DNA coding for the PelSS B esterase gene was also sequenced and the introns of the B esterases from the two strains compared. Differences at the amino acid level between the two esterases should contribute to the differences seen at the kinetic level.

A *Cx quinquefasciatus* strain, MRES, from Cuba was used to study the similarities between B₁ esterases. This strain is OP resistant and electrophoretically has a B₁ esterase. Bioassays and biochemical assays have shown that this strain also has an altered acetylcholinesterase-based OP resistance mechanism. The migration theory (Raymond *et al.*, 1991) suggests that amplification of the B₂ esterases (and possibly the B₁ esterase) has occurred once and subsequently spread around the world. The B₁ esterase cDNA was isolated from the MRES strain and compared with the sequence of the TEM-R B₁ esterase gene. Genomic studies on *Eco*RI digested MRES DNA were carried out to discover if the typical 2.1 kb fragment of the B₁ esterase gene haplotype (Raymond *et al.*, 1989) was the same in MRES and if the B₁ esterase gene from MRES is amplified when compared to the susceptible strain, PelSS. The results of these studies would determine whether the B₁ esterase genes from TEM-R and MRES had been amplified independently.

3.2 MATERIALS AND METHODS

3.2.1 Mosquito strains

The MRES strain of *Cx quinquefasciatus* is an OP and carbamate resistant strain from Havana and was collected from the Quibu River district in 1986. An altered acetylcholinesterase and an elevated B₁ esterase resulted in high levels (> 1000 fold) of malathion resistance in this strain (Bisset *et al.*, 1991). The strain was selected as fourth instar larvae at the 85%-95% mortality level for 22 generations

with malathion. Insecticide resistance has been maintained since by exposing the fourth instar larvae of every third generation to the LD₅₀ concentration of malathion. The PelSS strain has been described previously (Mosquito strains 2.2.1).

3.2.2 Methods

All methods used in this CHAPTER were similar to those previously used (METHODS 2.2). Using conventional PCR and the modified RACE procedures, these methods were used to isolate, clone and sequence cDNAs for the B₁ esterase from the OP resistant strain, MRES and the B esterase from the OP susceptible strain, PelSS. Using PCR with *Pfu* DNA polymerase (Stratagene) B esterase gene sequence was isolated, cloned and sequenced from the PelSS strain as it had been from PelRR, Tanga85, Dar91 and SPerm.

3.3 RESULTS

A full length cDNA coding for the MRES B₁ esterase was successfully isolated and sequenced using the above techniques. At least two PCR products were sequenced in each case and all overlapping PCR products were identical apart from two different 5' untranslated regions. As for the TEM-R and PelRR B esterase cDNAs, the MRES B₁ cDNA had a 1623 bp open reading frame which coded for a protein of 540 amino acids. The modified 5' RACE (METHODS 2.2.4.2) procedure was unsuccessful in isolating the 5' end of the PelSS B esterase cDNA. The partial length cDNA isolated for PelSS had an open reading frame of 1524 bp and coded for a partial protein of 503 amino acids. Both the B esterase cDNAs had a 3' untranslated sequence after the stop codon and a polyadenylation consensus sequence followed by a polyA(+) tail.

All the B esterase cDNA nucleotide sequences isolated to date (PelRR B₂, TEM-R B₁, MRES B₁ and PelSS B) were compared and this comparison is shown in FIGURE 3.1. The degree of identity between the four esterase cDNA open reading frames is shown in TABLE 3.1 and this includes the number of nucleotide differences between the four esterase cDNAs. The inferred amino acid sequence of the four esterases and that of a partial B₂ esterase amino acid sequence (Mouches *et al.*, 1990) are compared in FIGURE 3.2. Possible N-linked glycosylation sites are shown in FIGURE 3.2. The degree of identity between the five amino acid sequences is shown in TABLE 3.2 and this includes the number of amino acid differences between the five esterases.

The ratio of silent to non-silent nucleotide changes (those not leading to a change in amino acid sequence compared to those that do) can be used to determine whether a sequence is under active positive selection pressure. TABLE 3.3 shows this ratio as the number of silent nucleotide differences and the percentage of this number compared to the total number of nucleotide differences between the coding regions of the five B esterases. Where there are differences between the five esterases, there are only two different amino acids at any one residue. The number of amino acids unique to one sequence range from 1 (partial B₂) to 11 (TEM-R B₁) with PelRR B₂ (2), MRES B₁ (4) and PelSS (9) in between.

In Southern blot analysis, the B₁ esterase gene (from TEM-R) is contained within a 2.1 kb *EcoRI* fragment and the B₂ esterase gene (from all strains with this phenotype, including PelRR) is contained within a 9 kb *EcoRI* fragment (Raymond *et al.*, 1989; 1991; FIGURE 2.6). To determine if the MRES B₁ esterase gene conformed to either of the previously identified B₁ and B₂ esterase gene

FIGURE 3.1 continued

PelRR	210v	220v	230v	240v	250v	260v	270v	280v	290v	300v
TEM-R	TGCTATCACTtCGAtCGGCGCCTCCAGAAgATcGTcGGCTCGAGGACAGTCTGAAGATCAACAGTgTTTGGAAaGAGATCAACCCtTCAaagCCgCTTC									
MRES	*****t**c*****c*****c*****c*****c*****g*****g*****g*****cc**t*****									
PelSS	*****t**t*****t*****c*****c*****c*****c*****g*****g*****g*****ag**g*****									
PelRR	310v	320v	330v	340v	350v	360v	370v	380v	390v	400v
TEM-R	CGGTGATGCTGTACATtTACGGCGGtGGCTTCACGGAAGAACCGAAGCTGTACGGtCCGGATTtTcTTGGTTCAGAGGATATCGTtTTGGT									
MRES	*****c*****c*****g*****g*****g*****c*****c*****g*****cc*****g*****g*****									
PelSS	*****t*****t*****t*****t*****t*****t*****t*****t*****t*****t*****g*****g*****									
PelRR	410v	420v	430v	440v	450v	460v	470v	480v	490v	500v
TEM-R	GTCGTTCAACTACCGcATTGGGGCGTtGgGaTtCcTGTtTCAATCGGaGAGATGGCGTACCCGGTAATGCCGGACTCaAAGATCAGAACTTGGCC									
MRES	*****t*****t*****a**t**t*****a*****a*****a*****a*****a*****a*****a*****									
PelSS	*****c*****c*****g**a**c*****a*****t*****t*****c*****c*****c*****c*****									
PelRR	510v	520v	530v	540v	550v	560v	570v	580v	590v	600v
TEM-R	ATtCGgTGgGTTCTGGAGAAcATTGcCGCCtTTGGAGGgGACCCGAGCGCGTGACCCCTGgTcGgtCATAGCGCAGGTGCCGCTTCGGTgCAGTATCATC									
MRES	***c**t*****c*****c*****c*****a*****a*****a*****a*****a*****a*****a*****									
PelSS	***t*g*****g*****g*****g*****t*****t*****t*****t*****t*****t*****g*****g*****									
PelRR	610v	620v	630v	640v	650v	660v	670v	680v	690v	700v
TEM-R	TGATcTCGGATGcTtCCAAGGACTTGTtTCAGCGGGCTATcGTAAATGTCTGGAGTACGTATaAcAGTTGGTcGtTGACCCAGGCAACCTGGGTTGA									
MRES	***t*****g*****g*****g*****g*****g*****g*****g*****g*****g*****g*****									
PelSS	***c*****t*****t*****t*****t*****t*****t*****t*****t*****t*****t*****g*****g*****									
PelRR	710v	720v	730v	740v	750v	760v	770v	780v	790v	800v
TEM-R	GAGTTGCCGAAGCCATCGGTTGGGATGGACAGGGTGGTGAGTCCCGGtGCGTTGAGATtCTTGAAaAGCTGCCAAACCGGAGGACATtGTtGCTaACCAG									
MRES	*****g*****g*****g*****g*****g*****g*****g*****g*****g*****g*****c*****									
PelSS	*****t*****t*****t*****t*****t*****t*****t*****t*****t*****t*****a*****a*****									

FIGURE 3.1 continued

PelRR	810v	820v	830v	840v	850v	860v	870v	880v	890v	900v
TEM-R	GAGAA	GCTTCTG	ACTGAC	CAGGAC	ATATCT	TGACCG	TGACCG	TGACCG	TGACCG	ATtCCG
MRES	*****	g*****	*****	t*****	c*****	*****	*****	*****	*****	a*****
PelSS	*****	t*****	*****	c*****	*****	*****	*****	*****	*****	g*****
PelRR	910v	920v	930v	940v	950v	960v	970v	980v	990v	1000v
TEM-R	aaCCG	TtGAG	ATgGCT	CGAAct	GCTTGG	GGTGAC	AGATaG	ATATCAT	GATCGG	AGaACTTCT
MRES	ca***	c***g	*****	a*****	t*****	t*****	a*****	a*****	a*****	*****
PelSS	ag***	t***g	*****	t*****	t*****	a*****	g*****	g*****	g*****	*****
PelRR	aa***	t***a	*****	t*****	*****	a*****	*****	*****	*****	*****
PelRR	1010v	1020v	1030v	1040v	1050v	1060v	1070v	1080v	1090v	1100v
TEM-R	GCAaC	CGGAAC	TACTgT	CCCATC	CtCATgT	TCCTGG	GAATgT	TCCTCA	AACTTGA	AGATCAG
MRES	***t*	*****	g**c*	*****	t*****	t*****	*****	*****	*****	*****
PelSS	***a*	*****	g**g*	*****	*****	c*****	*****	*****	*****	*****
PelRR	1110v	1120v	1130v	1140v	1150v	1160v	1170v	1180v	1190v	1200v
TEM-R	AAGCA	AGTTACT	ACCCCG	ATAGC	AGTCC	TTCTG	ATGATG	CTCCG	CGGTCT	CTGCGC
MRES	**a**	*****	c***	t***	a***	a***	a***	a***	a***	a***
PelSS	**g**	*****	t***	g***	a***	a***	a***	a***	a***	a***
PelRR	1210v	1220v	1230v	1240v	1250v	1260v	1270v	1280v	1290v	1300v
TEM-R	CCATC	CTGCCCC	GCTCG	ATCGCG	CGCCG	CACCTT	CGTG	TACCG	GATCTG	CTGCGC
MRES	*****	t***	c***	*****	*****	*****	*****	*****	*****	*****
PelSS	*****	c***	a***	*****	*****	*****	c***	*****	*****	*****
PelRR	1310v	1320v	1330v	1340v	1350v	1360v	1370v	1380v	1390v	1400v
TEM-R	CCCGA	AGTGG	CGGAC	GCGCC	ATGCT	CTATCT	TTC	CAACTT	CAC	CaGTC
MRES	*****	*****	*****	*****	c***	t***	a***	*****	*****	*****
PelSS	*****	*****	*****	*****	c***	g**	c***	a***	a***	a***

FIGURE 3.1 continued

	1410v	1420v	1430v	1440v	1450v	1460v	1470v	1480v	1490v	1500v
Pe1RR	CTGCAACGCTG	TCGATGTG	TCACCGG	TTTCATCA	ACGGGATC	CAAACTGT	GCGATG	CGGTCG	GTGTG	TTCGAGCCGACgCGC
TEM-R	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
MRES	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
Pe1SS	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
	1510v	1520v	1530v	1540v	1550v	1560v	1570v	1580v	1590v	1600v
Pe1RR	AGACGAAGCC	ACGTTCA	AGTCTGA	ACATG	CCACGAC	GGGTCG	CGGTAT	ATCCG	GATG	CGGACCATGTA
TEM-R	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
MRES	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
Pe1SS	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
	1610v	1620v	1630v	1640v	1650v	1660v	1670v	1680v	1690v	1700v
Pe1RR	CGTGAACG	ATGAGCT	GTTTGAG	GGGAC	AGTTT	TAAAT	ATCCT	GTTG	AGAT	TGTTAgGttaaatacacATTTtATCaGt\$
TEM-R	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
MRES	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
Pe1SS	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
	1710v	1720v	1730v	1740v	1750v					
TEM-R	TgttgTt	tattt	taatt	atgtatt	GTGAATAA	TTATAct	t***C\$			
MRES	*tcata	*gacccg	-----	-----	-----	-----	-----	-----	-----	-----
Pe1SS	*tcata	*gact	-----	-----	-----	-----	-----	-----	-----	-----

TABLE 3.1 The numbers of nucleotide differences and the percentage identity between the coding regions of esterase B genes from five strains of *Culex quinquefasciatus*. The five strains are PelRR (esterase B₂), TEM-R (esterase B₁; Mouches *et al.*, 1990), MRES (esterase B₁) and PelSS (a partial length esterase B).

	PelRR B ₂	TEM-R B ₁	MRES B ₁	PelSS B
PelRR B ₂	*	95.7 %	98.2 %	98.0 %
TEM-R B ₁	70	*	95.2 %	95.2 %
MRES B ₁	30	78	*	98.8 %
PelSS B	31	73	18	*

The percentage identities are in the upper right-hand portion of the table and the nucleotide differences in the lower left-hand portion.

FIGURE 3.2 The identities between the inferred amino acid sequences of B esterases from five *Culex quinquefasciatus* strains. The strains were PelRR (esterase B₂), TEM-R (esterase B₁; Mouches *et al.*, 1990), MRES (esterase B₁), PelSS (esterase B) and a previously published amplified B₂ esterase (Raymond *et al.*, 1991). The amino acid sequence for PelRR esterase B₂ is shown in the top row. The same amino acid in the other esterases is shown by an asterisk (*) and the stop is represented by an X. The PelSS B esterase sequence starts at position 38 and the previously published esterase B₂ sequence starts at position 269 and ends at position 483. Lower case letters are used when one sequence differs from any other sequence. Possible N-linked glycosylation sequences are underlined. The three amino acids (S¹⁹¹, E³²⁴ and H⁴⁴²) which are thought to make up the active site triad are in bold type.

	10v	20v	30v	40v	50v	60v	70v	80v	90v	100v
PelRR	MSLESLTVQTKYGPVVRGKR	sVSLLGQEYVSFQGI	PYARAPEGELRFKAPVPPQ	nWTETLDCsQQCEPCYHf	DRRLQKI	VGCEDSLKIN	VFAKEIN	PSkPLP		
TEM-R	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
MRES	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
PelSS	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
	110v	120v	130v	140v	150v	160v	170v	180v	190v	200v
PelRR	VMLYIYGGFTEGTSGETLYGPD	FLVQKDIVLVSFN	YRIGALGFLCCQSeQD	GVPCGNAGLkDQNL	AIrWVLENI	aAFG	GDPKRVTL	vGHS	AGAA	SVQYHLI
TEM-R	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
MRES	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
PelSS	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
	210v	220v	230v	240v	250v	260v	270v	280v	290v	300v
PelRR	SDASKDLFQRAIVMSGSTYn	SWSLTRQRN	WVEKLAKAIGWD	QGGS	GALRFLkAAKP	EDIVAnQEKLLTDQ	DMQDDIF	TFPGPT	VEPYL	TEQCmIPKePF
TEM-R	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
MRES	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
PelSS	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
B ₂										

FIGURE 3.2 continued

	310v	320v	330v	340v	350v	360v	370v	380v	390v	400v
Pe1RR	EmARTAWGDk	IDIMIGTSEEG	LLQIKlqPELL	SHPh	FLGNvPPNL	KISMEKR	IEFAAKLQRY	YpDSSp	SMenNLGYV	HMSDRVFWHGLHRTILA
TEM-R	*m*****k	*****k	*****h	*****q	*****v	*****v	*****v	*****e	*****i	*****e
MRES	*m*****k	*****k	*****q	*****q	*****v	*****v	*****v	*****s	*****s	*****e
Pe1SS	*i*****k	*****k	*****q	*****q	*****i	*****i	*****a	*****s	*****s	*****a
B ₂	*m*****r	*****r	*****h	*****h	*****v	*****v	*****g	*****g	*****e	*****e
	410v	420v	430v	440v	450v	460v	470v	480v	490v	500v
Pe1RR	RaARSrARTFV	YRICLDSEFF	YNHYRIMMID	PKLRGTAHA	DElsYLFsn	FTQqVPgKe	TFEYRGLQTL	VDVftAFVING	DPNCgMTAK	SGVVFEPNaQTKPT
TEM-R	*a*****a	*****a	*****a	*****a	*****s	*****g	*****g	*****s	*****g	*****a
MRES	*a*****a	*****a	*****a	*****a	*****s	*****g	*****k	*****t	*****s	*****s
Pe1SS	*a*****a	*****a	*****a	*****a	*****f	*****l	*****k	*****t	*****s	*****s
B ₂	*g*****g	*****g	*****h	*****h	*****s	*****n	*****q	*****t	*****s	*****s
	510v	520v	530v	540v						
Pe1RR	FKCLNIANDG	vAfVDYPDA	DRlDMWDAM	YVNDELFX						
TEM-R	*****v	*f*****	*****	*****						
MRES	*****l	*l*****	*****	*****						
Pe1SS	*****l	*f*****	*****	*****						

TABLE 3.2 The numbers of amino acid differences and the percentage identity between the coding regions of esterase B genes from five strains of *Culex quinquefasciatus*. The five strains are PelRR (esterase B₂), TEM-R (esterase B₁; Mouches *et al.*, 1990), MRES (esterase B₁), PelSS (esterase B) and a previously published (Raymond *et al.*, 1991) partial length amplified esterase B₂.

	PelRR B ₂	TEM-R B ₁	MRES B ₁	PelSS B	B ₂
PelRR B ₂	*	97.4 %	98.0 %	97.0 %	99.1 %
TEM-R B ₁	14	*	96.1 %	95.2 %	97.7 %
MRES B ₁	11	21	*	97.6 %	99.1 %
PelSS B	15	24	12	*	95.8 %
B ₂	3	6	6	10	*

The percentage identities are in the upper right-hand portion of the table and the amino acid differences in then lower left-hand portion.

TABLE 3.3 The number of silent nucleotide differences (those not leading to a change in the amino acid sequence) and the percentage of this number compared to the total number of nucleotide differences between the coding regions of esterase B genes from four *Culex quinquefasciatus* strains. The four strains are PelRR (esterase B₂), TEM-R (esterase B₁; Mouches *et al.*, 1990), MRES (esterase B₁) and PelSS (a partial length esterase B).

	PelRR B ₂	TEM-R B ₁	MRES B ₁	PelSS B
PelRR B ₂	*	77.1%	63.3 %	51.6 %
TEM-R B ₁	54	*	70.5 %	64.4 %
MRES B ₁	19	55	*	22.2%
PelSS B	16	47	4	*

The percentage of silent nucleotide changes are in the upper right-hand portion and the number of nucleotides in the lower left-hand portion.

haplotypes and to look for gene amplification, a 1350 bp PelRR B₂ esterase cDNA fragment was hybridised to *Eco*RI digested genomic DNA from MRES and compared with genomic DNA from PelRR and PelSS. The resulting fragments are shown in FIGURE 3.3. The results from PelRR and PelSS have been discussed previously (CONCLUSIONS 2.4). The *Eco*RI fragments from the MRES strain, which electrophoretically has esterase B₁, differed from that reported for TEM-R. There was a fragment doublet of 3.2 and 3.0 kb (due to the reproducibility of the autoradiograph, this doublet is not obvious on FIGURE 3.3), which has not previously been reported for any B esterase. There was also a doublet of low intensity fragments at 3.6 and 3.7 kb (this doublet is not clearly visible in FIGURE 3.3 but was clearly visualised in the original autoradiograph).

To look at the relationship between genomic sequences of B esterase genes from resistant and susceptible *Cx quinquefasciatus* strains, a region of PelSS genomic DNA was cloned and sequenced. A similar region has already been isolated from PelRR and three more OP resistant strains with increased levels of B₂ esterase (FIGURE 2.5). The comparison between these sequences and that of the TEM-R B₁ esterase gene (including two introns) are shown in FIGURE 3.4 and the identities between the three sequences are shown in TABLE 3.4.

3.4 CONCLUSIONS

The B₁ esterase gene from the OP resistant TEM-R Californian strain of *Cx quinquefasciatus* (Mouches *et al.*, 1990) and a full length B₂ cDNA from the OP resistant *Cx quinquefasciatus* strain, PelRR (CHAPTER 2) have been cloned and sequenced. Increased B esterase activity causes resistance to OP insecticides in *Cx quinquefasciatus* mosquitoes and gene amplification of B₁ and B₂ esterase

FIGURE 3.3 Southern blot and hybridisation of digested genomic DNA from three *Culex quinquefasciatus* strains. The DNA was digested with *Eco*RI, separated on a 0.8% agarose gel, blotted and hybridised with a 1350 bp PelRR B₂ esterase cDNA probe. DNA samples are from the *Culex quinquefasciatus* strains PelRR (**Lane 1**), MRES (**Lane 2**) and PelSS (**Lane 3**). The final wash was in 0.1 x SSC and 0.1% (v/v) SDS at 60°C for 20 minutes. The blot was exposed to autoradiographic film overnight at -70°C with intensifying screens. The size of the fragments are shown in kilobase pairs (kb). Although not obvious on the **FIGURE**, the original autoradiograph clearly showed the presence of two doublet bands in MRES (**Lane 2**). The two doublets were at 3.2 kb and 3.0 kb and a fainter less intense pair of bands at 3.7 kb and 3.6 kb.

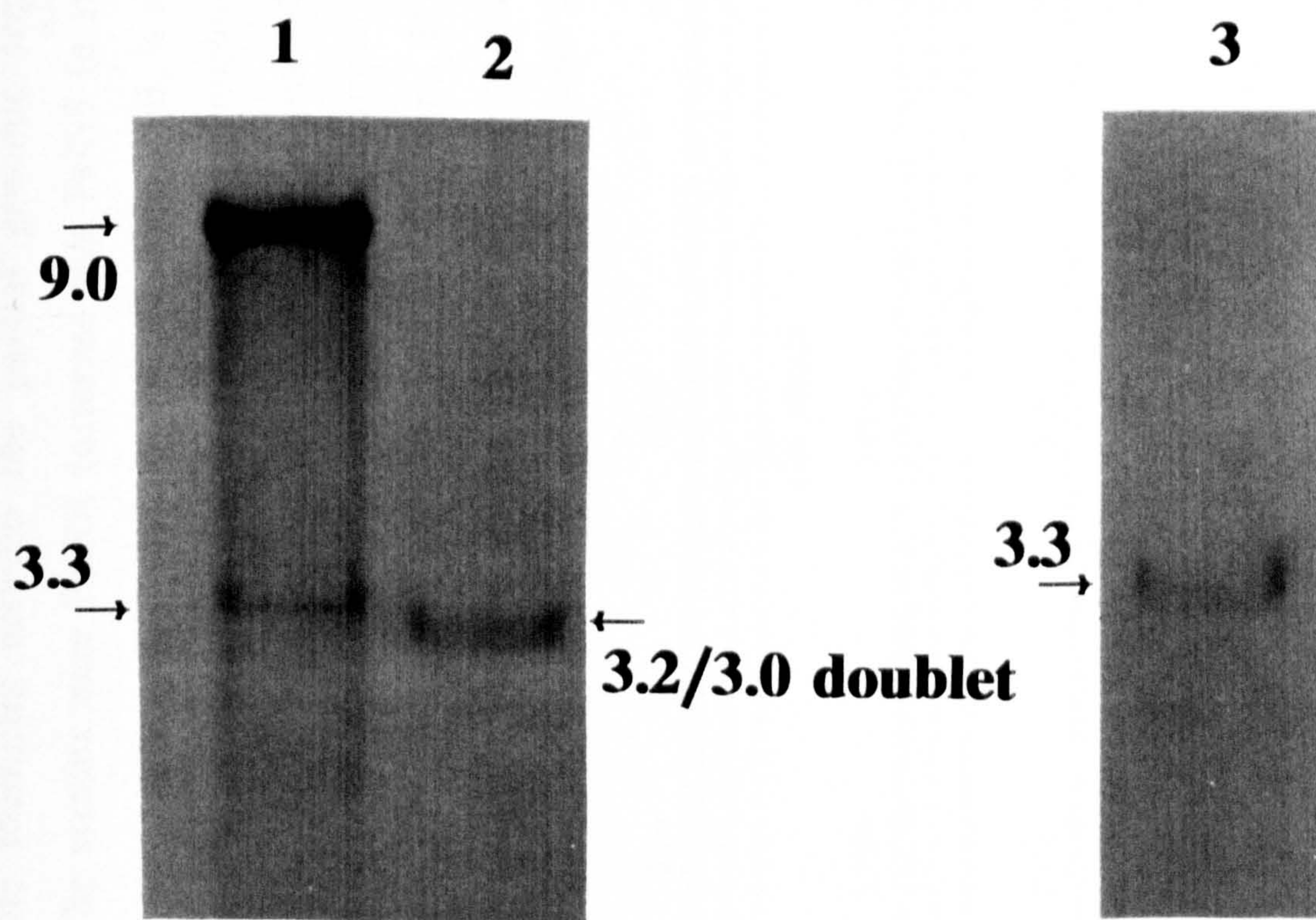


TABLE 3.4 The number of nucleotide differences and the percentage identity between the partial genomic sequences of the esterase B genes from three strains of *Culex quinquefasciatus*. The four strains are PelRR (esterase B₂), PelSS (esterase B) and TEM-R (esterase B₁; Mouches *et al.*, 1990). The 1182 bp region of DNA includes introns 2 and 3 from the TEM-R B₁ and their homologues from the PelRR B₂ and PelSS B esterase genes.

		PelRR B ₂	PelSS B	TEM-R B ₁
PelRR B ₂	exon	*	17	48
	intron		10	32
	whole		27	80
PelSS B	exon	98.4%	*	53
	intron	91.6%		23
	whole	97.8%		76
TEM-R B ₁	exon	95.5%	95.0%	*
	intron	80.7%	80.7%	
	whole	94.0%	93.6%	

Exon is just exon sequence, intron is just intron sequence and whole is the exon and intron sequence. The top right hand of the table shows the number of differences between the sequences when comparing the introns, the exons and the sequence as a whole. The bottom left hand of the table shows these differences as a percentage identity between the introns, exons and the whole.

genes is undoubtedly the cause of their increased expression.

In this study, a further B₁ esterase cDNA has been cloned and sequenced from an OP resistant strain of *Cx quinquefasciatus* from Cuba, MRES. A partial length cDNA for a B esterase from an OP susceptible *Cx quinquefasciatus* strain (PelSS) has also been isolated. The presence of a doublet of *Eco*RI fragments on a Southern hybridisation for the MRES B₁ esterase gene, whose total intensity is stronger than that of the PelSS *Eco*RI fragment, suggests that, as for TEM-R B₁ and PelRR B₂ esterase, increased activity of the esterase in the MRES strain is due to gene amplification.

As discussed earlier (CONCLUSIONS 2.4), PCR amplification of B esterase cDNAs, could result in the amplification of more than one allele. Sequencing of multiple overlapping PCR products suggested that for MRES, the slightly amplified B₁ esterase gene allele was isolated. The same procedure was carried out for PelSS, although the *Eco*RI pattern suggests that only a single allele is present in this strain due to the single 3.3 kb *Eco*RI fragment. The *Eco*RI patterns of the three strains (PelRR, PelSS and MRES) show that MRES has a unique, slightly amplified (when compared to PelSS) doublet of B esterase *Eco*RI fragments of 3.0 and 3.2 kb, as well as a low faint doublet of fragments at 3.6 and 3.7 kb. The MRES B₁ esterase gene amplification is therefore different from TEM-R B₁ and PelRR B₂. There is an *Eco*RI site (GAATTC) in the MRES B₁ esterase cDNA (and the other B esterase cDNAs also) which covers bases 98-103 of the open reading frame. The Southern blot of the genomic *Eco*RI digest was probed with a cDNA fragment that was downstream of this *Eco*RI site. The B₁ esterase gene from the TEM-R strain is about 3 kb in length and the fragments from the MRES

strain are greater than 3 kb. Therefore, it seems likely that the MRES B₁ esterase gene *Eco*RI fragment pattern was caused by the probe binding to two different genes and not to two halves of the same gene separated by an *Eco*RI restriction site. The two different 5' untranslated cDNA sequences found for the MRES B₁ esterase cDNA demonstrate the existence of two slightly different genes. Although the open reading frames attached to these two 5' untranslated regions were identical over 159 bp, the gene sequences in the 3' untranslated region could be of different lengths thereby giving the two *Eco*RI fragments. Therefore, as all the open reading frame sequences isolated were the same, it is probable that a gene coding for exactly the same protein has been amplified in two similar forms. It is interesting that a doublet of very low intensity fragments is also present in the MRES strain, although until analysis of further resistant strains is carried out, it will be difficult to determine the significance of this.

The high identities of the B₁ and B₂ esterases at the molecular level suggest that the esterase genes are allelic. The majority (70.5%, 55) of the nucleotide differences between the MRES B₁ and TEM-R B₁ esterase cDNAs are silent (they do not lead to a change in their amino acid sequences). There are however, 21 amino acid differences between the two proteins. Furthermore, the percentage identity of the amino acid sequence from PelRR B₂ esterase is 97.4% with TEM-R B₁ and 98.0% with MRES B₁, whereas TEM-R B₁ and MRES B₁ are 96.1% identical. In addition, the *Eco*RI fragments for the MRES B₁ esterase gene, are different from the 2.1 kb fragment of the TEM-R esterase B₁ gene (Raymond *et al.*, 1989). The differences between the two B₁ esterase genes suggest that they are different alleles which were amplified separately. Alternatively, if a single amplification event occurred, the genes have since diverged to a greater extent

than the electrophoretically distinct PelRR B₂ esterase has from either of the two B₁ esterases. The implication of this, coupled with the high percentage of silent to non-silent nucleotide changes, is that, if a single amplification event of the two B₁ esterases has occurred, it is not within the time span over which OPs have been used.

The changes that have occurred between the B esterase alleles are due to single nucleotide substitutions, which are scattered throughout the coding sequence. Hence differences must be due to point mutations that have accumulated over time. The present work shows that there are different alleles for electrophoretically identical B esterases, judged by nucleotide and inferred amino acid sequence. On the basis of this data and previous work (Poirie *et al.*, 1992), there are at least five B esterase alleles which have probably been independently amplified. There are 9 amino acid positions in the PelSS B esterase where the amino acid is different to that found in any resistance-associated B esterase. One or more of these amino acids may lead to the reduced binding of the oxon analogues of the OPs to the PelSS B compared to the amplified B esterases (Karunaratne, 1994). Conversely, amino acids unique to the B₁ and B₂ esterases may affect these binding rates. It is likely that the sequencing of more B esterase genes from strains which have already been shown to have kinetically distinct enzymes, will reveal further variants. Comparisons of a larger number of B esterases from susceptible and resistant strains will help to elucidate those residues essential for the increased binding of the oxons. If the B esterases can be expressed at high levels using suitable expression systems, point mutations can be introduced into the cDNA to study the effect of single and multiple amino acid changes on the kinetic characteristics of the expressed esterase with OP oxons. A

greater understanding of how the B esterases sequester the OPs and the residues involved will aid in the design of new insecticides that will not be detoxified by existing resistance associated esterases.

The partial genomic sequences isolated from PelRR, PelSS and TEM-R B esterases were very similar. The two introns sequenced from PelRR B₂ and PelSS B esterase started at the same place as those in the TEM-R B₁ esterase gene (introns 2 and 3). A higher degree of identity was seen between the PelRR and PelSS B esterase introns (91.6%) than either PelRR or PelSS showed with TEM-R (80.7% in both cases). A similar pattern (although less pronounced) was observed within the exon sequences (PelRR and PelSS, 98.4%; PelRR and PelSS with TEM-R, 95.5% and 95.0% respectively). Intron sequence is obviously not under as much selection pressure as exon sequence which would explain the greater degree of difference.

The fact that both the PelRR and PelSS strains arose from the same Sri Lankan parental colony, Pel, may explain why their B esterases are more similar to each other than to the TEM-R B₁ esterase, which was isolated from a Californian *Cx quinquefasciatus* strain. It is possible that the B₂ esterase gene was a product of the amplification and subsequent mutation of the PelSS B esterase gene. The initial amplification event can not have occurred recently though as there are significant differences between the open reading frames of PelRR and PelSS (31 nucleotides and 15 amino acids). However, since the B₂ esterase genes from around the world appear to be exactly the same (CHAPTER 2) and there is no evidence that the A₂/B₂ phenotype first arose in Sri Lanka, the degree of identity between the PelRR B₂ and PelSS B esterase cDNAs could be coincidental.

Alternatively, it is also possible that the B esterase gene found in PelSS may have co-migrated with the amplified B₂ esterase gene and replaced the existing B esterase gene population.

It has been suggested that a high level of variability exists in the B esterase genes not involved in resistance (Raymond *et al.*, 1991). However, the PelSS strain, despite being selected from multiple females, seems to have only one B esterase allele. As mentioned above, the *EcoRI* pattern seen for the PelSS B esterase gene could have been caused by the co-migration of a second B esterase with the amplified B₂ esterase. The variability (if any) in *EcoRI* patterns for the B esterase genes that are not highly amplified (if at all) in the other strains with the A₂/B₂ phenotype (Tanga85, Dar91 and SPerm) therefore needs to be determined and compared with the variability of field populations. B esterase gene RFLP patterns of field populations will determine the degree of variability of the B esterase gene.

A smaller number of differences are seen between the PelSS and MRES B esterase open reading frames (18 nucleotides and 12 amino acids) suggesting that these two esterases are also closely related. The comparative data suggests that the PelRR, PelSS and MRES B esterase genes (all nucleotide identities between the three are 98.0% or more) have all diverged from an ancestral allele at a different time than the TEM-R B₁ esterase gene (all nucleotide identities with the other three are 95.7% or lower). Further B esterase alleles will have to be sequenced to determine if the TEM-R B₁ esterase is isolated from the other B esterases presently sequenced.

CHAPTER 4

CHAPTER 4: THE CLONING AND SEQUENCING OF THE FIRST A ESTERASE cDNA FROM THE *CULEX QUINQUEFASCIATUS* MOSQUITO: ESTERASE A₂ FROM THE OP RESISTANT STRAIN, PELRR

This CHAPTER describes the isolation of the first *Cx quinquefasciatus* A esterase cDNA. N-terminal sequencing of the purified A₂ esterase from the *Cx quinquefasciatus* strain, PelRR, had been unsuccessful, so no amino-acid sequence for the protein was known. Therefore, with the use of an A₂ esterase anti-serum, a PelRR fourth instar larvae cDNA expression library was constructed and screened with the antiserum. RACE procedures were then used to isolate a probable full-length A₂ esterase cDNA.

4.1 INTRODUCTION

The OP resistant strain of *Cx quinquefasciatus*, PelRR from Sri Lanka, has elevated esterases A₂ and B₂ (Peiris & Hemingway, 1990a; 1990b). This is the most common esterase phenotype found associated with OP resistance in *Cx quinquefasciatus* mosquitoes. Previous work has shown that the elevation of the B₂ esterase (as well as the B₁ esterase) is due to gene amplification (Mouches *et al.*, 1986; 1990; Raymond *et al.*, 1989). The A₂ and B₂ esterases occur in complete linkage disequilibrium (the two esterases are always elevated together in a resistant individual). The A₂ and B₂ esterases have been purified and characterised kinetically for their interaction with a number of insecticides and resistance is conferred to the OPs by sequestration of the OPs oxon analogue by both the A₂ and B₂ esterases (Karunaratne, 1994). Recent work has shown an immunological cross-reactivity between the two esterases, although the anti-A₂

esterase antiserum showed 50 times less affinity for the B₂ esterase than the A₂ esterase (Karunaratne *et al.*, 1993b).

To compare the two esterases further, the A₂ esterase gene needed to be isolated. The esterases' kinetic, physical and immunological similarities (Karunaratne *et al.*, 1993b; Karunaratne, 1994) suggest that they have a similar protein structure. It was not clear however, if the A₂ esterase gene was amplified (although this seemed likely). Alternatively, the mRNA could be stabilised or there could be increased transcription and/or translation of the gene. To further study the A₂ esterase, a bacteriophage expression library was constructed from the fourth instar larvae of the PelRR *Cx quinquefasciatus* strain. This was immunoscreened with the anti-A₂ esterase antisera and using additional modified RACE procedures, a cDNA coding for the A₂ esterase was isolated. The cDNA was compared with that of the PelRR esterase B₂ at both the nucleotide and amino acid level to discover how closely related the genes were. To elucidate if amplification of the A₂ esterase gene was the probable underlying mechanism conferring resistance, a Southern blot of an *EcoRI* digest of genomic DNA from PelRR and PelSS was probed with the A₂ esterase cDNA.

Recent work carried out in our laboratory on the purification of the B₁ esterase from the *Cx quinquefasciatus* Cuban strain, MRES, suggested that there was also an over-expression of an A esterase in this strain, although this was not evident on electrophoresis of crude MRES homogenate. To study this further, a Southern blot of MRES *EcoRI* digested genomic DNA was also probed with the A₂ esterase cDNA to look for the amplification of an A esterase gene in this strain.

4.2 METHODS

4.2.1 Isolation of A₂ esterase cDNA

4.2.1.1 *PelRR cDNA library construction*

The cDNA for library construction from PelRR was prepared as in METHODS 2.2. However 5 µg of mRNA were used and all the other components of the reactions were scaled up accordingly. The cDNA was then resuspended in 38 µl of deionised water and heated to 68°C for 5 minutes. This denatured any double-stranded structures that may have formed from protruding single-stranded termini. In a 50 µl reaction at 37°C for 20 minutes the cDNA was blunted in T4 DNA polymerase repair buffer [18 mM (NH₄)₂SO₄, 66 mM Tris-HCl (pH 8.3), 6.6 mM MgCl₂, 10 mM β-mercaptoethanol], with the addition of 2 units of T4 DNA polymerase. The reaction was stopped by the addition of 1 µl of 0.5 M EDTA (pH 8.0). After phenol/chloroform extraction the cDNA pool was size selected with a Sephacryl S-400 column (Pharmacia). The S-400 column removed those cDNAs less than 400 bp in length. These cDNAs were not full length and their small size would interfere with the linker addition in the next step and the subsequent ligation into the bacteriophage arms of the vector. After ethanol precipitation of the size fractionated cDNA and resuspension in TET, 500 ng of the cDNA was used for linker addition. A vast excess of *Eco*RI linkers (Promega) were ligated to the cDNA overnight at 16°C in a 20 µl reaction in T4 DNA ligase buffer with 10 units of T4 DNA ligase. The linker reaction was stopped by heating to 70°C for 10 minutes and cooling on ice. The attached linkers were then phosphorylated in a kinase reaction. The reaction was carried out in 40 µl (including the 20 µl of the linker reaction) in kinase buffer [70 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 5 mM DTT, 0.01 mM ATP] with the addition of 20 units of T4 polynucleotide kinase (Promega) at 37°C for 30 minutes. After

phenol/chloroform extraction and a further size fractionation with Sephacryl S-400 (Pharmacia), to remove unligated linkers, the cDNA was ethanol precipitated and resuspended in 20 μ l of deionised water. The library was constructed in Lambda ZAP II (Stratagene). The bacteriophage arms were supplied digested with *Eco*RI and dephosphorylated. The advantage of this bacteriophage system, is that it allows the *in vivo* excision of the pBluescript phagemid containing the insert. This allows the recombinant cDNA to be characterised in a plasmid system which is far easier to manipulate than a bacteriophage. In the ligation reaction, 5 μ g of arms were ligated to 125 ng of cDNA overnight at 4°C with 10 units of T4 DNA ligase and then packaged using Packagene Extracts (Promega). Approximately 2×10^5 clones were obtained.

4.2.1.2 Immunoscreening with the *A*₂ esterase antiserum

This was carried out with the Protoblot Immunoscreening System (Promega). *E. coli* Y1090r- cells (0.6 ml of an overnight culture) were infected with the λ ZAP II bacteriophage and plated with LB soft agar [LB medium is 10 g of bacto-tryptone, 5 g of bacto-yeast extract and 10 g of NaCl in one litre of water. The pH was adjusted to 7.0 with NaOH and sterilized by autoclaving for 20 minutes at 15 pounds *per* square inch in on a liquid cycle. LB soft agar is prepared by adding 7 g of agar to the medium before autoclaving] the onto LB plates [LB plates are poured with LB agar which is LB medium with the addition of 15 g of agar *per* litre of medium prior to autoclaving]. Forty thousand pfu were used for each 150 mm plate. The plates were inverted and incubated for 3.5 hours at 42°C and then overlaid with a nitrocellulose disk (Hybond, Amersham) which had previously been saturated with 10 mM IPTG. The plates were then incubated at 37°C for a further 3 hours after which time the position of the filters was marked. The filters

were lifted and soaked in TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20] to remove any remnants of agar. To saturate non-specific binding sites, the filters were incubated in TBST + 1% BSA for 30 minutes. The filters were then incubated with TBST + the primary antibody (the A₂ esterase antiserum) for 30 minutes followed by three washes with TBST for 10 minutes each. The filters were then transferred to TBST + the secondary antibody-alkaline phosphatase conjugate at a 1:7500 dilution (the secondary antibody is an anti-rabbit antibody and binds specifically to the first antibody, enabling detection of those bacteriophage expressing protein which the anti-A₂ antiserum binds to) and incubated for 30 minutes. After three more washes for 10 minutes in TBST the filters were blotted and transferred to colour development substrate solution [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl₂]. For each 10 mls of the colour development substrate solution, 66 µl of NBT (nitro blue tetrazolium, 50 mgs/ml in 70% dimethylformamide) and 33 µl of BCIP (5-bromo-4-chloro-3-indolyl phosphate, 50 mgs/ml in dimethylformamide, were added. Positive clones appeared as purple plaques (this is due to the alkaline phosphatase attached to the second antibody reacting with the NBT and BCIP to give a purple product) and colour development was stopped by soaking the filters in Stop Solution [20 mM Tris-HCl (pH 8.0), 5 mM EDTA]. To retest a putative positive signal, an agar plug was removed from the position on the plate corresponding to the purple plaque on the filter. The plug was incubated for at least one hour in 1 ml of SM, gently vortexing occasionally. The bacteriophage were then replated and the screening procedure repeated until all the plaques on the plate produced a positive signal.

4.2.1.3 *In vivo* excision of the pBluescript plasmid from the LAMBDA ZAP II vector

DNA sequences in the λ ZAP II vector and the presence of f1 bacteriophage-derived proteins allow the *in vivo* excision of any cloned insert contained within the vector. In a 50 ml conical tube 200 μ l of *E. coli* XL1-Blue cells ($OD_{600} = 1.0$), 200 μ l Lambda ZAP II bacteriophage stock ($> 10^5$ pfu) and 1 μ l of R408 helper bacteriophage ($> 10^6$ pfu/ml) were added and incubated for 15 minutes at 37°C. Five millilitres of 2 x YT medium [16 g of bacto-tryptone, 10 g of bacto-yeast extract and 5 g of NaCl in one litre of water. The pH was adjusted to 7.0 and the medium was sterilized by autoclaving for 20 minutes at 15 pounds *per* square inch on a liquid cycle] were added and incubated for 3 hours at 37°C with shaking. The tube was heated to 70°C for 20 minutes and centrifuged at 4000g for 5 minutes. The supernatant contains the pBluescript phagemid stock. To rescue this phagemid, 10 μ l of phagemid stock was added to 200 μ l of *E. coli* XL1-Blue host cells ($OD_{600} = 1$) and incubated for 15 minutes at 37°C. One hundred microlitres of this was then plated on an LB/Ampicillin plate [LB ampicillin plates are poured using LB agar with the addition of 1 ml of 50 mg/ml ampicillin to each litre of molten LB agar, cooled to 50°C] and incubated overnight, inverted, at 37°C. The colonies which grew contained recombinant pBluescript plasmids with an insert coding for a protein recognised by the A₂ esterase antiserum.

4.2.1.4 Exonuclease III/Mung Bean Nuclease Deletion

Exonuclease III/mung bean nuclease deletion was used to produce unidirectional deletions of the positive A₂ esterase cDNA plasmid clone (pBlueAV.A2) from the PelRR cDNA library screening and followed the instructions supplied with the kit (Stratagene). pBlueAV.A2 was digested with a unique 3'-overhang restriction enzyme (*SacI*) and a unique 5' or blunt restriction site (*XbaI*), which was between

the 3' restriction site and the insert. The DNA was treated with exonuclease III (Stratagene) and at time intervals, aliquots were removed. Exonuclease III digests 3' ends from blunt ends or 5' overhangs and thus digested the DNA unidirectionally into the insert site from the *Xba*I site. This left an undigested single strand overhang. This DNA was then completely digested with mung bean nuclease. The blunt ends created with the mung bean nuclease were then recircularised with T4 DNA ligase and transformed into competent *E. coli* JM109 cells (Promega). This procedure created a series of unidirectional deletions of the insert DNA.

4.2.1.5 *Modified 5' RACE*

The PelRR A₂ esterase cDNA isolated and sequenced from the λ ZAP II cDNA library was thought not to be full length and thus the modified 5' RACE procedure, as performed for the B esterases (METHODS 2.2), was used to isolate the full length A₂ cDNA. The reverse primer used in the PCR was 5' ACCGTACATCTCCACTCC 3' and was close to the 5' end of the cDNA isolated from the cDNA library.

4.2.2 *DNA sequencing*

The subcloning of the 5' RACE PCR fragments and the sequencing of the various plasmids containing the PelRR A₂ esterase cDNA was as before (METHODS 2.2). Two 5' RACE products (coding for the 5' end of the A₂ esterase cDNA) were sequenced in both directions using M13 universal primers and synthesised internal primers. The Exonuclease III/Mung Bean Nuclease Deletion (METHODS 4.2.1.4) was used to sequence the pBlueAV.A2 insert in one direction and internal primers were used to sequence the opposite strand.

4.2.3 Haplotype analysis

Southern blot hybridisation analysis was performed as before (METHODS 2.2) using genomic DNA from PelRR, PelSS and MRES. The probe used was the insert from pBlueAV.A2.

4.2.4 Phylogenetic tree construction

The amino acid sequence of A₂ esterase was compared with the *Cx quinquefasciatus* B esterases and all other known insect carboxylesterases and a phylogenetic tree was constructed. *T. californica* AChE was also included in the alignment, since its three dimensional structure is known and an alignment between this structure with the three dimensional structure of *G. candidum* lipase has been carried out (Cygler *et al.*, 1993). All other known carboxylesterase sequences were superimposed onto this alignment and a phylogenetic tree of *T. californica* AChE and *D. melanogaster* AChE ought to show that the two AChEs are related. The sequences were aligned by the Clustal V method (Higgins & Sharp, 1989). In this method, the sequences are aligned in larger and larger groups. Initially, a crude similarity measure between every pair of sequences is calculated, using an approximate alignment algorithm (Wilbur & Lipman, 1983). These scores are then used to calculate a 'guide tree' or dendrogram, which will decide the order in which the sequences are aligned in the final multiple alignment. The dendrogram is calculated using the UPGMA method (Sneath & Sokal, 1973). Once the dendrogram has been calculated, the sequences are aligned in larger and larger groups using the algorithm of Myers & Miller (1988) for the optimal alignments. The final phylogenetic tree is constructed by neighbourhood joining (Saitou & Nei, 1987). The method works on a matrix of distances between all pairs of sequences to be analyzed. These distances are related to the degree

of divergence between the sequences. The final tree displays the distance between sequence pairs as a percentage divergence (100% minus percentage identity). The tree is given an arbitrary root, although it is not truly rooted (to correctly root a tree, a known ancestral sequence forms part of the alignment).

4.3 RESULTS

The initial screening of the 0.2×10^6 recombinant clones from the unamplified PelRR cDNA library with the anti-A₂ esterase antiserum gave four positive plaques. The four plaques were purified, *in vivo* excised and partially sequenced with M13 Forward and Reverse primers. The partial nucleotide sequences of all four clones (200 bp with the M13 Forward and Reverse primers) were identical. Therefore the insert from just one of the plasmids (pBlueAV.A2) was completely sequenced in both directions.

The cDNA sequence (from pBlueAV.A2) had an open reading frame at its 5' end and terminated in a stop codon, 3' untranslated region with a polyadenylation signal and a polyA(+) tail. There was no start methionine codon (AUG), so a modified 5' RACE procedure (METHODS 2.2: 4.2) was used to isolate the 5' end of the cDNA. The sequence of the two subcloned 5' RACE PCR products were identical in both directions and also overlapped exactly with the previously sequenced partial length cDNA clone, pBlueAV.A2. The full length cDNA (made up of the insert from pBlueAV.A2 and the 5' RACE PCR product) had an open reading frame of 1623 bp complete with an AUG start codon. There was an in frame stop codon (TGA) upstream from the AUG start codon, which is good evidence for the suggested methionine being the amino terminal of the protein. The open reading frame coded for a protein of 540 amino acids and this is in the

expected range as the purified monomeric protein has an estimated molecular weight of 58-67 kDa as estimated by SDS PAGE, native PAGE and Sephacryl S-200 chromatography (Karunaratne, 1994). FIGURE 4.1 shows the full length PelRR A₂ cDNA nucleotide sequence and the predicted amino acid sequence. The probable residues involved in the active site triad (S¹⁹¹, E³²⁴ and H⁴⁴⁵) are shown and the two possible N-linked glycosylation sites, conforming to the sequence Asn-Xxx-Thr or Asn-Xxx-Ser, where Xxx is not proline, are underlined in FIGURE 4.1. One of the sites is very close to the active site serine (S¹⁹¹). FIGURE 4.2 shows the predicted amino acid sequence of PelRR esterase A₂ and its alignment with PelRR esterase B₂, which is always co-elevated with A₂ esterase. The two esterases share a 49.2% similarity. To further compare the A₂ esterase amino acid sequence with other carboxylesterases (including the B esterases) a phylogenetic tree was constructed which contained all insect carboxylesterases (and *T. californica* AChE) for which amino acid sequence was known. FIGURE 4.3 shows this tree. As expected, the tree groups the *Drosophila* esterases together and the *Cx quinquefasciatus* A and B esterases together. The two AChEs also group together, whereas the *M. persicae* E4 esterase and the *H. virescens* juvenile hormone esterase are on separate branches on the tree. The percentage divergence between any two sequences can be calculated by adding the total distances of the horizontal branches between the sequences.

The insert from pBlueAV.A2 was used as a probe for Southern blot analysis of *Eco*RI restriction digests of equal amounts of genomic DNA from the insecticide susceptible (PelSS) and resistant (PelRR) mosquito strains as well as the B₁ esterase expressing strain, MRES. The result of this hybridisation is shown in FIGURE 4.4. The PelRR strain had an amplified (when compared to PelSS) *Eco*RI

FIGURE 4.1 continued

510v 530v 550v 570v 590v
CCTCGCTCTCCGCTGGGTGGACAACGTCGCCAACTTTGGGGGTGACCCGAAGAACATTACGCTGTTGGAGAGAGTGCCGGGGTGTTCCTCGTGCCTACC
L A L R W V V D N V A N F G G D P K N I T L F G E S A G G C S V H Y
170^ 190^

610v 630v 650v 670v 690v
ACATGGTGTCCGACCTTTCGCGGGGATTTTCAGCGTCCGATCGTAATGTCCGGCTGTGTGCTGAACAATTGGTCCGTGTTCCACGAAGAAAGTTTAGCGAA
H M V S D L S R G L F Q R A I V M S G C V L N W S V P R R K F S E
210^ 230^

710v 730v 750v 770v 790v
CGGCTGGCCAAAGCTCTCGGATGGAACGGGCGAGGTGGTGAGCGAGCTGCTCTGGAGGTGCTCGTGAAGCGGGATCCGGAGAGTATCGTTCGTGAGCAGGAAGT
R L A K A L G W N G Q G E R A A L E V L V K A D P E S I V R E Q E V
250^

810v 830v 850v 870v 890v
TTTGTTGAATGAGATGAGATCGAAATCGCATCCTATTTTCGTTTGGACCAGTGATTGAGCCCTACATTACAAAAAATGCATGATTCCAAAGATCCTGTAG
L L N E N E I E N R I L F S F G P V I E P Y I T K K C M I P K D P V
290^

930v 950v 970v 990v 1010v
AGATGTGTCGTGAAGCTTGGAGTAACGAAATCGACATTTTGATCGGGGAAACTCGGAAGAGGGTCTATTCTGTCTGAACGGGATCAAAGAAATCCTTCGATC
E M C R E A W S N E I D I L I G G N S E E G L F C L N G I K E N P S I
310^ 330^

1030v 1050v 1070v 1090v 1110v
ATGAGTAACTTGAAGGACTTTGAGTATCTGGTTCGCTGGAGCTGGACTTGGTACGCACATCTCAGAGATGCAAGAGGTGGCAAGCAGATGAAAAAGTTCTA
M S N L K D F E Y L V P L E L D L V R T S Q R C K E V G K Q M K K F Y
350^ 370^

1130v 1150v 1170v 1190v 1210v
CTACGGTGAACGGAACCGTCGTTTGAGAAATCGAGAGGGTTACCTTACGCTGATGACCGACAAGCTATTCCTGCACGGACTGCATCGTACGATCCTGTCAAGAC
Y G E T E P S F E N R E G Y L T L M T D K L F L H G L H R T I L S R
390^

1230v 1250v 1270v 1290v 1310v
TCAACTCGAAGAACCGTCGAAGACGTTCCGTACCGGTTCTCGGTAGATTTCGGACACCTACCACTACCGGATCGTGTTCCTGCGACAAGACGTGCGCGGA
L N S K K P S K T F L Y R F S V D S D T Y N H Y R I V F C D K N V R G
410^ 430^

FIGURE 4.1 continued

1350v 1370v 1390v 1410v
ACTGCCCATGCCGACGATCTGTCTGTACATCTTCAAGAACGTGTTCACAGATCCCCCGCGAAGGATACCTTCGAGCATCGTGGCATGATGAACATGGTCGGCTT
T A H A D D L S Y I F K N V F N D P P A K D T F E H R A M M N M V G L
450^ 470^

1450v 1470v 1490v 1510v 1530v
ATTCTCAACGTTCCGCGAGTAACAATGGAATCCCAACGGAGAGCAGATCAACGAATGGGAATCGATTGCGACGCCGCGGACCGTTCAAGTGTCTCAACATCA
F S T F A S N N G N P N G E Q I N E W E S I A T P A G P F K C L N I
490^ 510^

1550v 1570v 1590v 1610v 1630v
ACAACGACGGTTTGCAGTTTATTGAGTATCCGGAGCAGGAACGAATGAAGTTTGGGATTCACCTGTACAGCAAGATAAGCTTTATTAGGGATCATTAAGTGCAT
N N D G L Q F I E Y P E Q E R M K F W D S L Y S K D K L Y X
530^

1650v 1670v 1690v 1710v 1730v
TACAAATACAAATTATTGTAGTTTAAACTGTTCAATTTTGCTGCCCTGCAGCCAGGCAGATTGTTCGGGGTTGAGGGTGTCACTGAATAAATAACACGTT

1750v
AGATTCAACGCTTATCACCC\$

FIGURE 4.2 A comparison of the amino acid sequences of the amplified esterases A₂ and B₂ from the OP resistant *Culex quinquefasciatus* strain, PelRR. The two esterases are 540 amino acids in length. Boxed amino acids are shared by both esterases and to maximise the alignment, gaps (-) were introduced. The stop codon is indicated by a dot (.).

A2	M D V E H P V V P T Q Y G P V R G V R K L A A T G V D Y Y S F Q R I P Y V Q P P V G E L E F K D A O	50
B2	M S L E S L T V O T K Y G P V R G K R S V S L L G Q E Y V S F Q G I P Y A R A P E G E L R F K A P V	50
A2	P R K P W T E P L D C T V Q G P G G Y Q Y S K L I N K T I G R E D S L H M N V F T K N L D S K O L L	100
B2	P P O N W T E T L D C S O Q C E P C Y H F D R R L O K I V G C E D S L K I N V F A K E I N P S K P L	100
A2	P V M L Y I H G G A F M R G S S G V E M Y G P D Y L I O K D V V F V S F N Y R I G A L G F I S F D S	150
B2	P V M L Y I Y G G G F T E G T S G T E L Y G P D F L V O K D I V L V S F N Y R I G A L G F L C C O S	150
A2	P E L G L P G N A G L K D O N L A L R W V V D N V A N F G G D P K N I T L F G E S A G G C S V H Y H	200
B2	E O D G V P G N A G L K D O N L A I R W V L E N I A A F G G D P K R V T L V G H S A G A A S V O Y H	200
A2	M V S D L S R G L F O R A I V M S G C V L N N W S V V P R R K F S E R L A K A L G W N G O G G E R A	250
B2	L I S D A S K D L F O R A I V M S G S T Y N S W S L T R O R N W V E K L A K A I G W D G O G G E S G	250
A2	A L E V L V K A D P E S I V R E O E V L L N E N E I E N R I L F S F G P V I E P Y I T K K C M I P K	300
B2	A L R F L K A A K P E D I V A N O E K L L T D O D M O D D I F T P F G P T V E P Y L T E O C M I P K	300
A2	D P V E M C R E A W S N E I D I L I G G N S E E G L F C L N G I K E N P S I M S N L K D F E Y L V P	350
B2	E P F E M A R T A W G D K I D I M I G G T S E E G L L L O K I K L O P E L L S H P H L F - - L G N	348
A2	L E L D L V R T S O R C K E V G K O M K K F Y Y G E T E P S F E N R E G Y L T L M T D K L F L H G L	400
B2	V P P N L K I S M E K R I E F A A K L K O R Y Y P D S S P S M E N N L G Y V H M M S D R V F W H G L	398
A2	H R T I L S R L N S K K P S K T F L Y R F S V D S D T Y N H Y R I V F C K N V R G T A H A D D L S	450
B2	H R T I L A R - A A R S R A R T F V Y R I C L D S E F Y N H Y R I M M I P K L R G T A H A D E L S	447
A2	Y I F K N V F N D P P A K D T F E H R A M M N M V G L F S T F A S N N G N P N G E O I N E W E S I A	500
B2	Y L F S N F T Q O V P G K E T F E Y R G L O T L V D V E T A F V I N - G D P N C G M T A K S G V V F	496
A2	T P A G P - - - - F K C L N I N N D G L O F I E Y P E O E R M K F W D S L Y S K O K L Y .	541
B2	E P N A Q T K P T E K C L N I A N D G V A F V D Y P D A D R L D M W D A M Y V N D E L F .	541

FIGURE 4.3 The phylogenetic tree of all known insect carboxylesterase amino acid sequences. The tree was constructed using the Clustal V multiple alignment method (Higgins & Sharp, 1989). The scale represents the percentage divergence between the sequences. This divergence can be calculated by adding the distances of all the horizontal branches between any two sequences. *Torpedo californica* AChE was included in the tree construction, since its three dimensional structure is known.

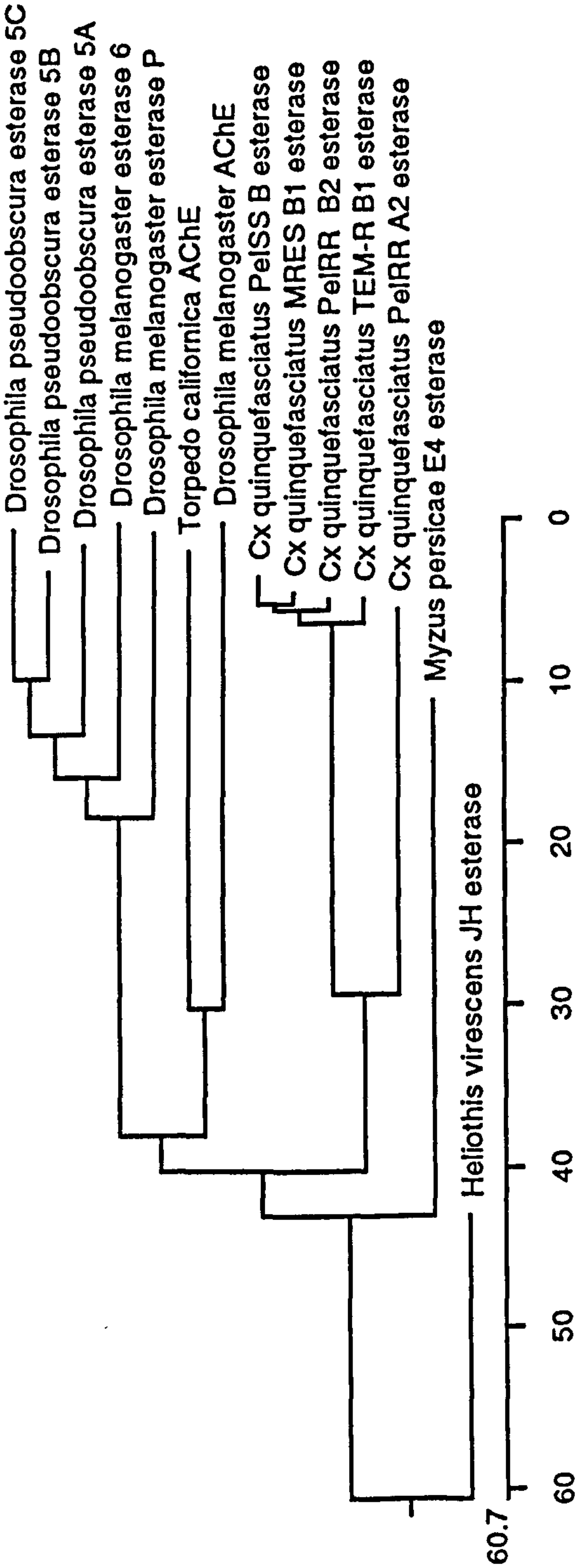
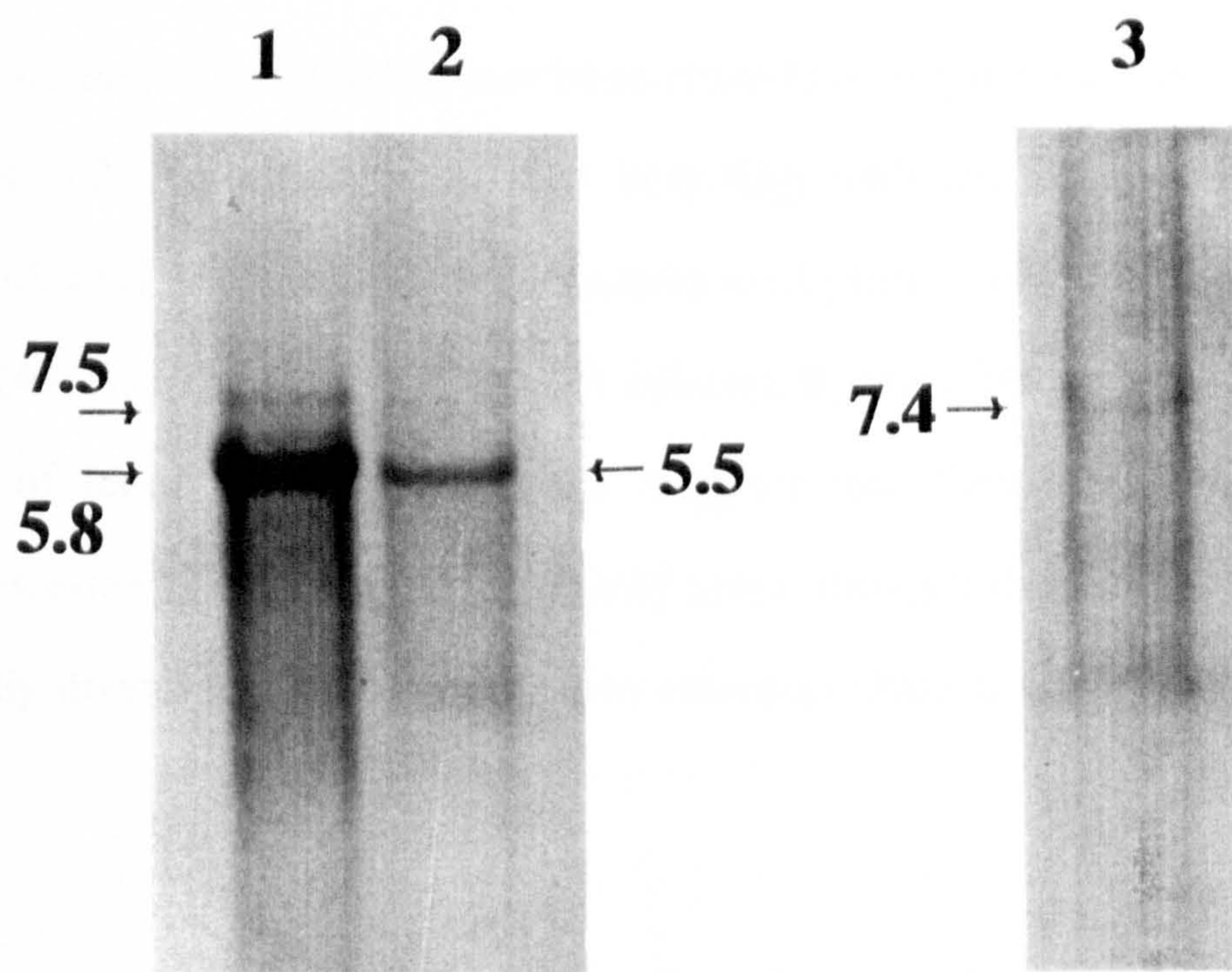


FIGURE 4.4 Southern blot and hybridisation of PelRR, PelSS and MRES digested genomic DNA. The genomic DNA was digested with *EcoRI*, separated on a 0.8% agarose gel, blotted and hybridised with the insert from the plasmid pBlueAV.A2, containing the partial length A₂ esterase cDNA. The *Culex quinquefasciatus* strains used were PelRR (**Lane 1**), MRES (**Lane 2**) and PelSS (**Lane 3**). The final wash was in 0.1 x SSC and 0.1% (v/v) SDS at 60°C for 20 minutes. The blot was exposed to autoradiographic film overnight at -70°C with intensifying screens. The size of the fragments are shown in kilobase pairs (kb). Although not obvious in the **FIGURE**, a weak band of 7.4 kb is present in MRES (**Lane 2**) which was clear in the original autoradiograph. The original autoradiograph also clearly showed the presence of only one band in PelSS (**Lane 3**) of 7.4 kb and a faint band of 6.4 kb in PelRR (**Lane 1**).



A₂ esterase gene fragment of 5.8 kb and just above this, a low intensity fragment of 6.4 kb (this is not obvious in FIGURE 4.4, although the band was clear on the original autoradiograph) and a further low intensity fragment at 7.5 kb. The MRES strain had an amplified (when compared to PelSS) band of 5.5 kb and a low intensity fragment of 7.4 kb (this band is not obvious in Figure 4.4, although the band was clear on the original autoradiograph). The PelSS strain had a single low intensity band of 7.4 kb (other bands below in FIGURE 4.4 are due to the reproduction of the autoradiograph).

4.4 CONCLUSIONS

A full length A₂ esterase cDNA from the insecticide resistant PelRR strain of the *Cx quinquefasciatus* mosquito has now been cloned and sequenced. The cDNA has an ORF of 1623 bp which has a high homology with the ORF of PelRR B₂ (58.3% similarity) and codes for a 540 amino acid protein which also has a high homology (49.2% similarity) with PelRR esterase B₂ (also 540 amino acids). The high level of identity between the two suggests that they originated from a common ancestor. The two genes probably arose through duplication and have subsequently diversified, although the two esterases share a number of common features.

Amino terminal signal sequences (usually of about 20 amino acids) are hydrophobic in nature and their presence causes a mature protein to be secreted (and possibly glycosylated) from the cell. All sequenced human liver carboxylesterases, acetylcholinesterases and the aphid E4 carboxylesterase (involved in sequestration in insecticide resistant aphids) contain signal sequences. The PelRR A₂ and B₂ esterases do not have signal sequences, suggesting that they

are not exported from the cell and neither of the purified esterases bind to Con A chromatography columns (Karunaratne, 1994), which suggests that they are not glycosylated. In contrast, the aphid esterase is glycosylated (Devonshire *et al.*, 1986b; Field *et al.*, 1993). Of the two possible glycosylation sites of the A₂ esterase, one is only four amino acids from the active site serine (S¹⁹¹) and the other, thirty-one amino acids from the serine. It is thus, extremely unlikely that the A₂ esterase is glycosylated.

Southern blot analysis showed that the A₂ esterase gene from the PelRR resistant strain had an amplified 5.8 kb *EcoRI* fragment, when compared to the susceptible strain, which had a single *EcoRI* fragment of low intensity of 7.4 kb. This result suggests that the underlying mechanism of elevated esterase A₂ activity is gene amplification, as it is for the B esterases. The elevated A₂ esterase and the corresponding non-elevated A esterase have been purified and kinetically characterised from both the PelRR and PelSS strains (Karunaratne, 1994). The two A esterases are significantly different in their interaction with the oxons of a number of OP insecticides. The A₂ esterase from PelRR binds more rapidly to the oxons than the A esterase from PelSS. Based on both biochemical and molecular biological studies, it is therefore clear that the OP resistant mechanism in the PelRR strain is more than likely to involve the amplification of A and B esterases that are different from those in a susceptible strain from the same location.

The MRES strain of *Cx quinquefasciatus* also has an amplified (when compared to PelSS) *EcoRI* fragment for an A esterase gene, at 5.5 kb. Recent protein purification studies in our laboratory have suggested the presence of an increased

amount of A esterase in this strain and the amplification of an A esterase gene in MRES adds weight to this finding. The *EcoRI* genomic fragment for the B₁ (RESULTS 3.3) and A esterase genes from the MRES strain are different from the fragments for the B₂ (RESULTS 3.4) and A₂ esterase genes from PelRR. Therefore, the amplification events that lead to the increase in copy number of the A and B esterase genes in the MRES strain probably occurred independently of the esterase gene amplification in the PelRR strain. Alternatively, the amplification events occurred such a long time ago as to allow for mutations leading to the different *EcoRI* fragments and sequence data (between MRES B₁ and PelRR B₂ esterases).

The low intensity *EcoRI* fragment from the PelSS strain for the B esterase gene (3.3 kb) was also seen in the PelRR strain at a similar intensity (RESULTS 3.4). In the case of the A esterase, the PelSS strain has a unique low intensity fragment of 7.4 kb compared to two bands of 7.5 kb and 6.4 kb in PelRR. It was suggested (CONCLUSIONS 3.4) that the similarity of the low intensity fragment for the B esterase gene in the PelRR and PelSS strains was due to the same gene being present in both strains. However, the low intensity A esterase gene fragments are different between the two strains. This could be due to the presence of different genes in the two strains, or the presence of the same gene with a different *EcoRI* fragment. Using PCR methodology it will be difficult to see if the two strains share the same low copy number (probably only one) gene, due to the presence of the vastly amplified A₂ esterase gene in the PelRR strain, which would probably make up the majority of any PCR product. This problem could be overcome by screening genomic libraries of the two strains (PelRR and PelSS) with A₂ esterase cDNA probes. DNA isolated from positive plaques could be

digested with *EcoRI* and those samples which gave the characteristic *EcoRI* fragment pattern for the gene not linked to resistance (the gene that di not give a 5.8 kb *EcoRI* fragment on Southern blot analysis) could be sequenced and compared. Significantly different open reading frames would imply that the two strains had different A esterase genes not linked to resistance. The MRES strain has a low intensity 7.4 kb *EcoRI* fragment (as does PelSS). Using the same experimental design as outlined above, this gene could also be isolated and sequenced. The present study has shown the presence of two amplified and at least two low copy number (probably one copy) A esterase genes in only three strains of *Cx quinquefasciatus*. It is likely that, as for the B esterases, more A esterase alleles will be found when further resistant *Cx quinquefasciatus* strains are studied.

The phylogenetic tree of the insect esterases demonstrates that the *Cx quinquefasciatus* A and B esterases are more closely related to each other than to other esterases. It has already been suggested that the *D. melanogaster* esterases, Est6 and EstP arose from a tandem duplication which probably occurred between 17 and 37 million years ago (Collet *et al.*, 1990). The phylogenetic tree shows this, since EstP and Est6 arise from a single node on the phylogenetic tree (divergence between the pair is 37.6%). Similarly, the *Cx quinquefasciatus* A and B esterases could also have arisen by a gene duplication, although the branches from the node connecting the two esterases is longer than for Est6 and EstP (divergence for A₂ and B₂ esterase is 48.7%).

CHAPTER 5

CHAPTER 5: GENOMIC ORGANISATION OF THE PELRR A₂ ESTERASE GENE AND THE ISOLATION OF FURTHER A ESTERASE GENE SEQUENCE

This CHAPTER describes the genomic organisation of the *Cx quinquefasciatus* A₂ esterase gene from PelRR. The easiest way to accomplish this was to construct a PelRR genomic library and to screen for recombinant clones containing the A₂ esterase gene. The success of this enabled the complete sequencing of the A₂ esterase gene and the discovery that it was contained within the same amplicon as the B₂ esterase gene.

5.1 INTRODUCTION

It is known that the amplified B₁ esterase gene from the *Cx quinquefasciatus* strain, TEM-R, is situated within an amplicon of about 25 kb (Mouches *et al.*, 1990). The gene has three introns and four exons and codes for a protein of 540 amino acids. It has been shown that the PelRR B₂ esterase gene has the typical amplified *EcoRI* fragment of 9 kb seen in a number of strains with an amplified B₂ esterase (CHAPTER 2; Raymond *et al.*, 1991). The B₂ esterase gene is one of at least five elevated B esterase alleles seen in *Cx quinquefasciatus* (Raymond *et al.*, 1989; 1991; Poirie *et al.*, 1992; Vaughan *et al.*, 1995). Introns 2 and 3 of the B₂ esterase gene are in exactly the same location as they are in the B₁ esterase gene (Mouches *et al.*, 1990) and the same is also true of the PelSS B esterase gene (CHAPTER 3). The existence of intron 1 in the PelRR B₂ and PelSS B esterases has yet to be shown. The PelRR A₂ esterase gene has an amplified (when compared to PelSS) *EcoRI* fragment of 5.8 kb. The A₂ and B₂ esterase

genes occur in complete linkage disequilibrium (the two esterases are always co-elevated in an individual resistant mosquito). It is likely therefore that the two esterase genes (A_2 and B_2) sit on the same amplicon because this situation would ensure that an individual would always possess both amplified genes. However, it is possible that the two genes sit on two separate amplicons which are very tightly linked and some published classical genetic data suggests that this might be the case (Wirth *et al.*, 1990).

To further study the A_2 esterase gene, a PelRR genomic library was constructed in λ GEM-12 (Promega) and probed with an A_2 esterase cDNA probe (the insert from pBlueAV.A2; CHAPTER 4). The presence of a large number of positive plaques would provide further evidence for amplification of the A_2 esterase gene. The A_2 and B_2 esterase proteins have a percentage similarity of 49.2% and probably arose from divergence after duplication of an ancestral gene. If there are introns in the A_2 esterase gene in identical locations to the B_2 esterase gene this will add weight to the hypothesis.

If the migration theory is correct (Raymond *et al.*, 1991), after a single amplification event, the A_2/B_2 esterase phenotype has spread quickly and is now found worldwide in *Cx quinquefasciatus* and *Cx pipiens* populations. It is not clear why this phenotype has become so dominant a resistance mechanism compared to the B_1 esterase amplification, particularly as we know that the MRES B_1 esterase gene may also be co-amplified with an A esterase gene (CHAPTER 4). There is a possibility that other active genes are also located on the amplicon(s) on which the A_2 and B_2 esterases are found. These genes could give a selective advantage to the A_2/B_2 esterase phenotype and explain its dominance. Sequencing

either side of the A₂ esterase gene was therefore undertaken to determine whether other genes were located nearby.

The B₁ and B₂ esterase cDNA sequences from MRES and PelRR respectively are different from each other (CHAPTER 3). At present, the only A esterase gene sequence known, is that of A₂ esterase from PelRR. The amplified *EcoRI* genomic fragment patterns of the MRES and PelRR A esterase genes are different (CHAPTER 4) and this could reflect differences in the gene sequences of the A esterases between the two strains, as it does for the B esterases. If a large number of gene sequences for the A esterase alleles from resistant and susceptible strains are determined, for which their inhibition kinetics are known, it should be possible to determine which particular amino acid residues of the esterases are essential for efficient sequestration of OPs. Since it has been shown that the MRES strain of *Cx quinquefasciatus* has an amplified A esterase gene (CHAPTER 4), primers used for the internal sequencing of the PelRR A₂ esterase gene were used in PCR reactions in an attempt to isolate A esterase gene sequence from MRES genomic DNA, in order to characterise a further amplified A esterase.

5.2 METHODS

5.2.1 Isolation of A₂ esterase genomic DNA

5.2.1.1 Construction of a PelRR genomic library

Genomic DNA was isolated from PelRR as described (METHODS 2.2). The λGEM-12 vector (Promega) was used for the library construction as it has six unique restriction sites in which to insert and excise the genomic DNA. The λGEM-12 *XhoI* Half-Site Arms kit (Promega) was used. The cloning strategy used

relied on the high specificity with which partially filled-in *Xho*I digested arms were specifically ligated to partially filled in *Sau*3A-I digested genomic DNA. The partial fill-in prevented self-ligation of arms and inserts and thus genomic DNA size fractionation was unnecessary and the number of non-recombinants was essentially zero. Ten micrograms of genomic DNA were partially digested with *Sau*3A-I so the majority of DNA pieces were between 15 and 23 kb (the range of sizes that would ligate into the λ GEM-12 arms). After phenol/chloroform and chloroform extraction, the digested genomic DNA was ethanol precipitated and resuspended in TET. In a 10 μ l ligation, 1 μ g of λ GEM-12 arms were ligated to approximately 0.15 μ g of partially digested PelRR genomic DNA overnight at 4°C with 10 units of T4 DNA ligase. The ligated DNA was packaged using Packagene Extracts (Promega). Approximately 0.5×10^6 clones were obtained.

5.2.1.2 Screening the PelRR genomic library

To isolate genomic DNA containing the A₂ esterase gene, the PelRR genomic library was screened with the A₂ esterase cDNA obtained from screening the PelRR cDNA library. *E. coli* LE392 cells (0.6 ml of an overnight culture) were infected with the λ GEM-12 bacteriophage and plated with LB soft agar onto LB plates (50000 pfu for each 150 mm plate). The plates were inverted and incubated for about 5 hours at 37°C. After the bacteriophage had just become visible, the plates were stored at 4°C for at least an hour to ensure hardening of the top agar. Duplicate lifts from each plate were carried out with Hybond N⁺ nylon filters (Amersham) and their position marked. The filters were denatured, fixed and then hybridised with a ³²P-labelled A₂ esterase cDNA probe (specific activity > 2×10^6 cpm/ μ g) at 65°C for 16 hours in hybridisation buffer [5 x Denhardt's solution, 6 x SSC, 0.1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate, 5% (w/v)

PEG 8000 and 100 $\mu\text{g/ml}$ boiled, sheared herring sperm DNA]. The final washes were at 65°C in 2 x SSC for 20 minutes. After autoradiography at -70°C for 5 hours with intensifying screens, positive plaques (those seen on both filters) appeared as small black dots. To retest a putative positive signal, an agar plug was removed from the position on the plate corresponding to the black dot on the filter. The plug was incubated for at least one hour in 1 ml of SM [20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM MgSO_4 , 0.01% (w/v) gelatin], with gentle, occasional vortexing. The bacteriophage were then replated and the screening procedure repeated until all the plaques on the plate produced a positive signal.

5.2.1.3 Preparation of bacteriophage lambda DNA

An overnight culture of *E. coli* LE392 was grown up by inoculating a single colony into 5 ml of LB media supplemented with 50 μl of 1 M MgSO_4 and 50 μl of 20% (w/v) maltose and rotating overnight at 225 rpm at 37°C. Six hundred microlitres of this overnight culture were added to 3×10^6 pfus of the positive bacteriophage stock. After 20 minutes at 37°C the inoculum was added to 75 ml of prewarmed (37°C) LB medium supplemented with 750 μl of 1 M MgSO_4 in a 250 ml Erlenmeyer flask. After overnight rotation at 225 rpm and 37°C, the bacteriophage concentration was very high due to repeated cycles of infection, reproduction and lysis of *E. coli* LE392 cells by the bacteriophage. After the addition of 1 ml of chloroform and further agitation for 30 minutes, the cells were sedimented at 5000g for 10 minutes and the supernatant was removed. RNase A and DNase I were both added to the supernatant to a concentration of 2 $\mu\text{g/ml}$, which was then incubated at 37°C for 30 minutes to digest any *E. coli* RNA and DNA present in the supernatant. The bacteriophage DNA is protected by its coat. For every 10 ml of supernatant 0.58 g of NaCl and 0.93 g of PEG 8000 were

added, dissolved and the mixture stored on ice for at least one hour. The combination of PEG and NaCl cause the bacteriophage to precipitate. The bacteriophage were recovered by centrifuging at 10000g for 20 minutes at 4°C and resuspended in 7.5 ml of SM with gentle vortexing and centrifuged at 8000g for 2 minutes at 4°C to remove any remaining cellular debris. To the supernatant was added 78 µl of 10% SDS and 78 µl of 0.5 M EDTA (pH 8.0). After heating to 65°C for 15 minutes (to release the DNA from the bacteriophage), the mixture was put on ice and extracted twice with an equal volume of phenol/chloroform and once with an equal volume of chloroform. An equal volume of isopropanol was added to precipitate the DNA and after an hour at -20°C the DNA was pelleted by centrifugation at 12000g for 20 minutes at 4°C. The DNA was washed with 7.5 ml of 70% (w/v) ethanol and resuspended in 150 µl of TET. Approximately 375 µg of bacteriophage DNA was isolated (5 µg for every initial ml of LB medium).

5.2.1.4 *Subcloning of recombinant bacteriophage DNA*

The DNA from each positive bacteriophage clone was doubly digested with a number of restriction enzymes. One of the pair was always *SacI*, as this site is within the vector in the multiple cloning site and digesting recombinant DNA with *SacI* will lead to the removal of the bacteriophage arms. The fragments were separated on a 0.8% agarose gel and transferred to nylon membranes. There is a *BamHI* restriction site (GGATCC) in the A₂ esterase cDNA at positions 800-805 (FIGURE 4.1). The insert from pBlueAV.A2 was cut into two fragments with *BamHI* to use as probes. The 5' probe (5'AV.A2) contained sequence up to the *BamHI* site in the cDNA and the 3' probe (3'AV.A2) contained sequence 3' to the *BamHI* site. The nylon membranes were hybridised with the two cDNA

probes and genomic fragments representing the 5' and 3' ends of the A₂ esterase gene were subcloned into the pBluescript plasmid (Stratagene). The inserts were partially sequenced using primers complementary to the pBluescript plasmid (M13 universal primers). Primers were then synthesised based on this initial sequence data to sequence further into the inserts. Sufficient primers were synthesised to enable the sequencing of both strands of the A₂ esterase gene.

5.2.2 Isolation of MRES A esterase genomic DNA

MRES genomic DNA was isolated (METHODS 2.2.6) and used as the template in PCR reactions to isolate a partial A esterase gene. The procedure (PCR reaction and subsequent subcloning) was carried out as before (METHODS 2.2.8) with a primer pair which was complementary to the PelRR A₂ esterase sequence. The primer pair used was 5' TGGGTGGTGGACAACGTC 3' and 5' AGAATAGACCCTCTTCCG 3'.

5.2.3 Sequencing of subcloned genomic DNA

Sequencing was undertaken using the ALF Automatic Sequencer (Pharmacia) as described before (METHODS 2.2) and a contiguous map for the whole A₂ esterase gene with sequence in both directions was constructed using the DNASTAR package (LASERGENE).

5.3 RESULTS

After screening 0.25×10^6 recombinant clones of the PelRR genomic library with the A₂ esterase cDNA probe, a very large number of positive plaques were seen. There were at least 20 strong positives on each of the five plates of 50000 recombinant clones. Four positives plaques were further processed to purity and

DNA from each recombinant bacteriophage clone was isolated. A double digest of one of the positive bacteriophage clones (bacteriophage A2.1) with *SacI* and *BamHI* digested a *SacI* fragment of 10 kb into two *SacI/BamHI* fragments of 6 kb and 4 kb. After Southern blotting and hybridisation, the 5'AV.A2 probe hybridised to the 6 kb *SacI/BamHI* fragment and the 3'AV.A2 probe hybridised to the 4 kb *SacI/BamHI* fragment. It was assumed that these two genomic fragments each contained the two halves of the A₂ esterase gene, separated by the *BamHI* site located in the A₂ esterase cDNA. The two fragments were subcloned into pBluescript digested with *SacI* and *BamHI* and sequenced with M13 universal primers through the *BamHI* site. The sequence through the *BamHI* cloning site of these two clones was identical to that from pBlueAV.A2. The plasmids were named pBlue5'AV.A2 (the 5' end of the A₂ esterase gene, up to the *BamHI* site) and pBlue3'AV.A2 (the 3' end of the A₂ esterase gene, from the *BamHI* site). Internal primers were synthesised to sequence the two halves of the gene in both directions. A 3069 bp stretch of DNA containing the whole PelRR A₂ esterase gene is shown in FIGURE 5.1. The gene was compared with the full length A₂ esterase cDNA previously sequenced (FIGURE 4.1). The positions of the introns were assumed from this comparison and the sites of the six introns and seven exons (as well as the corresponding amino acid sequence) are shown in FIGURE 5.1. The putative polyadenylation signal and the start of polyadenylation in the mature gene transcript are also shown. The *BamHI* site (GGATCC) which separates pBlue5'AV.A2 and pBlue3'AV.A2 is at position 1280 of the A₂ esterase gene. In order to compare the position of the introns in the A and B esterases, a comparison of the amino acid sequences of TEM-R B₁ esterase and PelRR A₂ esterase is outlined in FIGURE 5.2, along with their exon/intron boundaries. All the exon/intron boundaries present in the B₁ esterase gene were also present in

FIGURE 5.1 The complete nucleotide sequence and predicted amino acid sequence of the amplified A₂ esterase gene from the OP resistant strain of *Culex quinquefasciatus*, PelRR. The amplified gene was isolated from a genomic library screened with the insert from pBlueAV.A2. Positions of the exon/intron boundaries were based on the previously isolated A₂ esterase cDNA. The consensus site for polyadenylation (AATAAA) is marked and the polyA(+) addition site (with H).

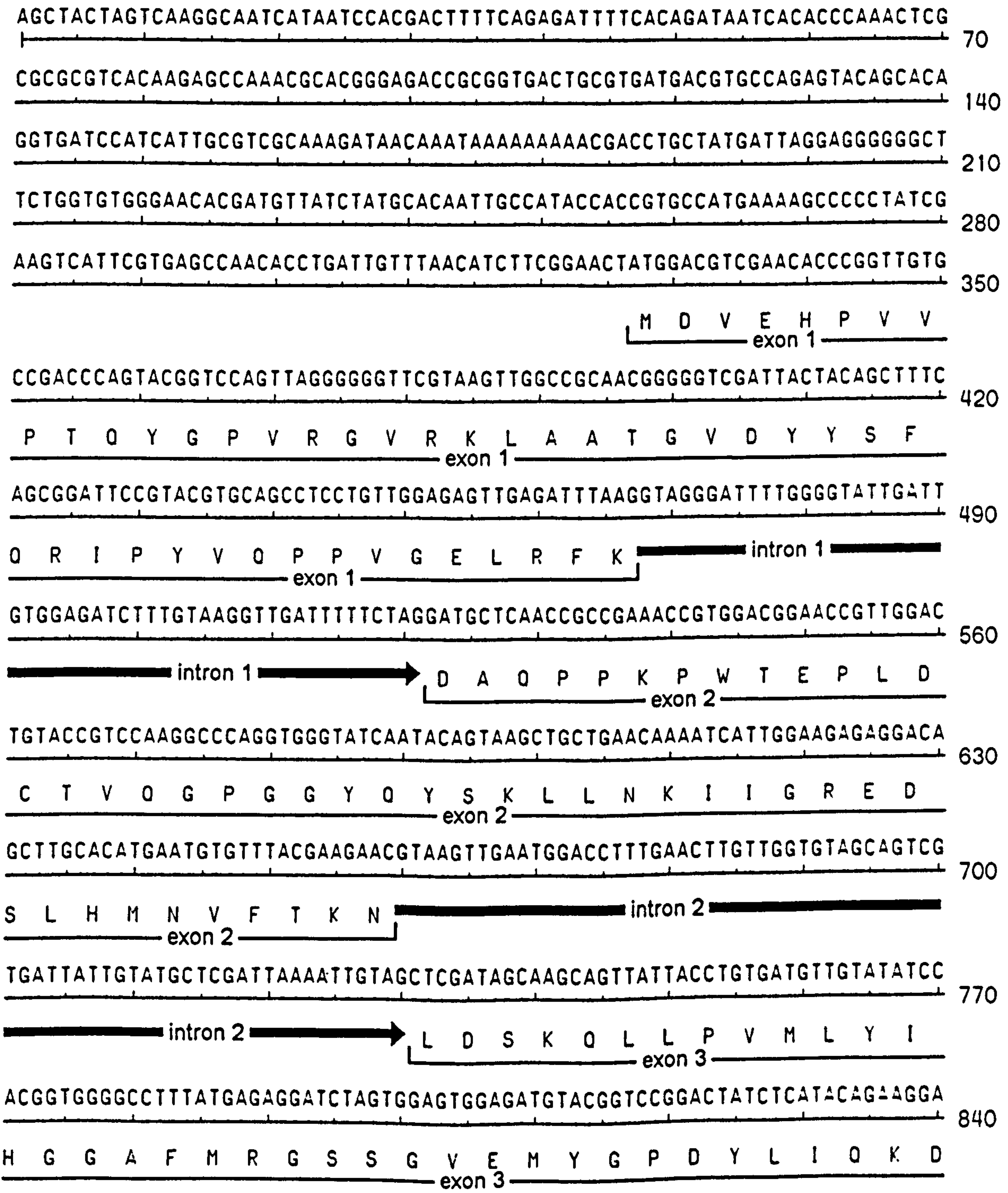


FIGURE 5.1 continued

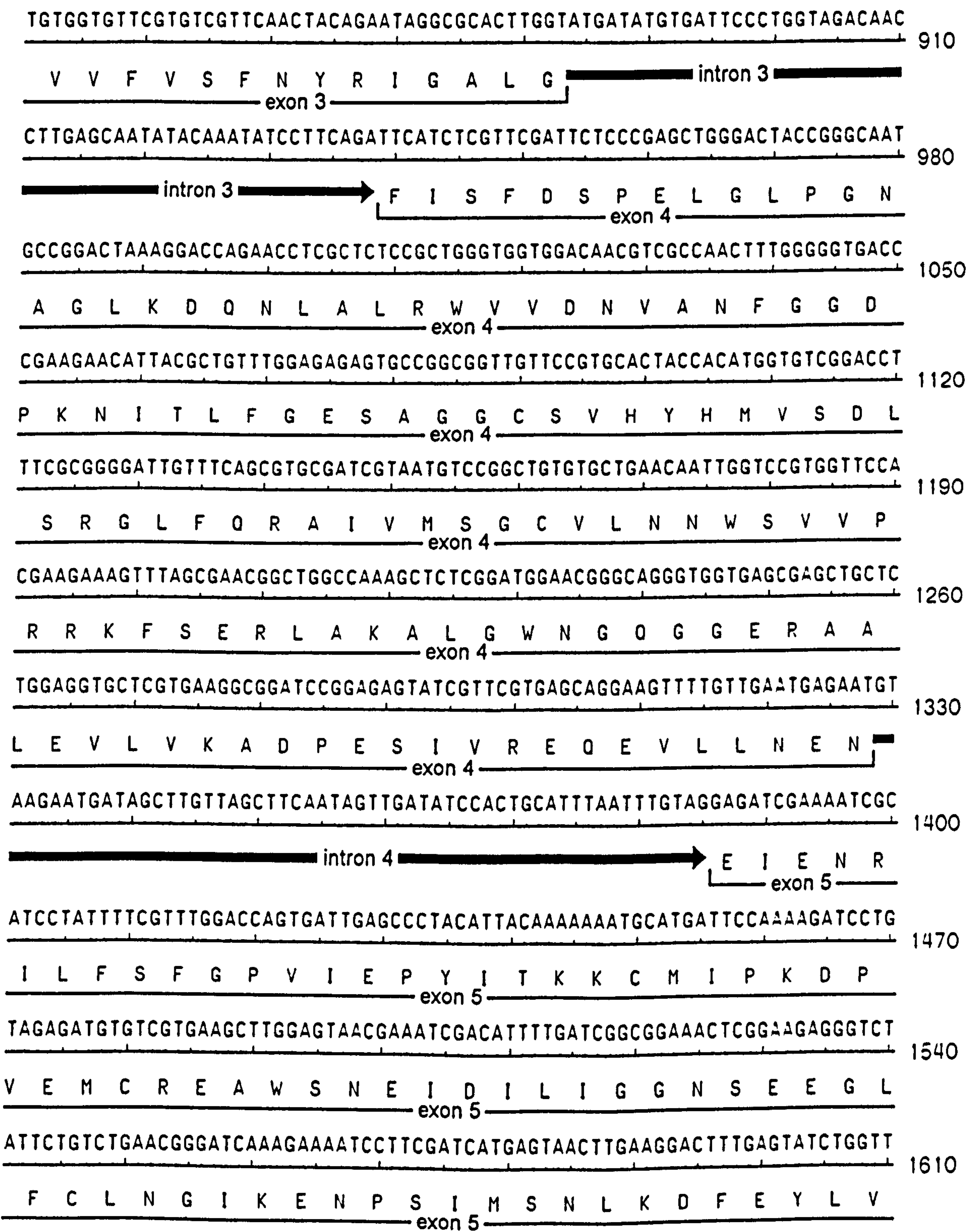


FIGURE 5.1 continued

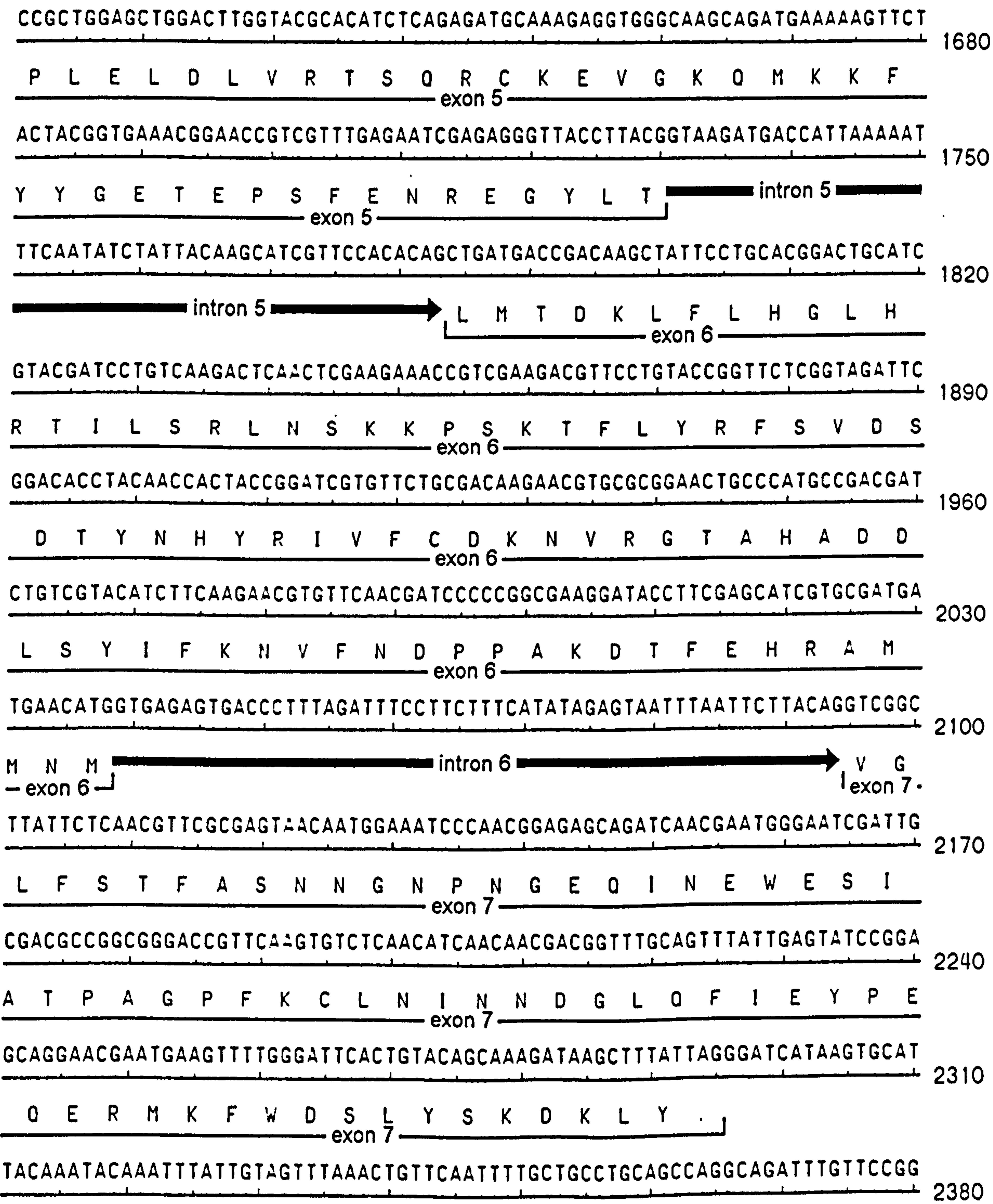


FIGURE 5.1 continued



FIGURE 5.2 An amino acid alignment of *Culex quinquefasciatus* TEM-R B₁ esterase (Mouches *et al.*, 1990) and PelRR A₂ esterase. The position of the introns in the two esterases are marked by a red vertical line between the two amino acids in which the intron occurs. TEM-R B₁ esterase has four exons and three introns and PelRR A₂ esterase has seven exons and six introns.

A2	MDVEHPVVP	TQYGPVRGV	RKLAATG	VDYYSFQRI	PYVQPP	40										
B1	MSLES	LTQTKYGPVRGK	RNVSL	LGQEYVS	FQGI	PYARAP	40									
A2	VGELE	FKDAQPP	KPWTEP	LDCTV	QGGYQYSKL	LNKI	IG	80								
B1	EGELR	FKAPVP	PQKWTET	LDCTQ	QCEPCY	HFDRRL	QKI	VG	80							
A2	REDSL	HMN	VFTKN	LD	SKQL	LPV	MLYI	HGGAF	MRGS	SGVEM	120					
B1	CEDSL	KI	NVFAKE	I	NPSTP	LPV	MLYI	YGGGF	TEGT	SGTEL	120					
A2	YGP	DYLI	QKD	VVF	VSF	NYRI	GALGF	I	SFDS	PELGL	P	GNAG	160			
B1	YGP	DFL	VQKDI	VL	VSF	NYRI	GALGF	L	CCQS	EQDGV	P	GNAG	160			
A2	LKDQNL	AL	RWV	VDN	VAN	F	GGDPKNI	TLF	GES	AGGC	S	VH	YH	200		
B1	LKDQNL	AI	RWV	LENI	AA	F	GGDPKRV	TLA	GHS	AGA	AS	VQ	YH	200		
A2	MVSD	LSRGL	FQRAI	VMSG	CVL	NNWS	VVPR	RKFS	ERL	AKAL	240					
B1	LISD	ASKDL	FQRAI	VMSG	STYSS	WSL	TRQR	NWVE	KL	AKAI	240					
A2	GWN	GQGGER	AALE	V	L	VKAD	PESI	VRE	QEV	LLNENE	I	ENRI	280			
B1	GWD	GQGGES	GALR	F	L	RAAK	PEDI	VAH	QEK	LLT	DQDM	QDDI	280			
A2	LFS	F	GPVI	EPYI	TKK	CM	PKDP	VEMC	REAWS	NEI	DI	LI	GG	320		
B1	FTP	F	GPTV	EPYL	TEQC	I	PKAP	FEMAR	TAWG	DKI	DI	MI	GG	320		
A2	NSEEG	LFC	L	NGI	KEN	PSI	MSNL	KDFE	YL	VPLE	LDL	V	RTSQ	360		
B1	TSEEG	LLL	L	QKI	KLH	PELL	SHPHL	F	-	-	LGN	VPPN	L	KISME	358	
A2	RCKE	V	GKQM	KKF	YYGETE	PSF	ENRE	G	YLTL	MTDKL	FL	HGL	400			
B1	KRIE	F	AAKL	KQRY	YPDSI	PSMEN	NLGY	VH	MMS	DRVF	WHGL	398				
A2	HRTI	LS	R	LNS	KKPSK	TFL	YRFS	VDS	DTYN	HRYI	VFC	DKNV	440			
B1	HRTI	LAR	-	AARS	RARTF	VYRI	CLDS	E	FYN	HRYI	MMI	DPKL	437			
A2	RGT	AHAD	DL	SYI	FKN	VFN	DP	PAK	DTFE	H	RAMM	NM	VGL	FST	480	
B1	RGT	AHADE	L	SYL	FSN	FTQQ	VP	GKET	FEYR	GLQ	TLV	DV	FSA	477		
A2	FAS	NN	G	NPN	GEQI	NE	-	-	-	WESI	ATP	AGPF	FKCL	NI	NNDGL	516
B1	FVI	N	-	GDP	NC	GMTAK	GGV	VFEP	NAQT	KPTF	FKCL	NI	A	NDGV	516	
A2	QFI	E	YPE	QER	MKF	WDS	L	YSK	DKLY	541						
B1	AFV	D	YP	DADR	L	DMWD	AMY	VN	DEL	F	541					

the A₂ esterase gene (which also had an additional 3 introns).

To search for further open reading frames within the A₂ esterase gene amplicon, partial sequence through the *SacI* site of both pBlue5'AV.A2 and pBlue3'AV.A2 was obtained using universal M13 primers. Open reading frames were found in both instances. The *SacI* site in the plasmid pBlue5'AV.A2 originated from the vector, λ GEM-12 and therefore is not present in the genomic DNA. Sequence after the vector nucleotides was identical to that of the 3' end of the PelRR B₂ esterase gene. The sequence was reading from 3' to 5' and therefore the 6 kb plasmid, pBlue5'AV.A2, can be assumed to contain virtually the whole of the B₂ esterase gene. The plasmid also contains the 5' end of the A₂ esterase gene, running from 5' to 3' (if the *SacI* site is assumed to be the 5' end). The plasmid therefore contains two partial genes (B₂ and A₂ esterase) which are in a head to head arrangement. Sequencing through the *SacI* site of pBlue3'AV.A2 gave an open reading frame also. This DNA sequence was subjected to the Blastx program at the NIH, USA. Blastx compares a query nucleotide sequence translated in all six possible reading frames to a protein sequence database. The DNA sequence showed high homology to the enzyme xanthine dehydrogenase (XDH) from a number of organisms. FIGURE 5.3A shows the DNA sequence and the predicted amino acid sequence and FIGURE 5.3B shows this amino acid sequence compared to partial XDH amino acid sequences from two insect counterparts, the fruit fly *D. pseudoobscura* and the bluebottle fly *Calliphora vicina*. A map of the insert from bacteriophage A2.1 showing the presence of the full length PelRR A₂ esterase gene and its relationship with the partial length B₂ esterase and XDH genes is shown in FIGURE 5.4.

FIGURE 5.3 The nucleotide sequence (346 nucleotides) and predicted amino acid sequence (A) of an open reading frame (115 amino acids) located 3' to the PelRR A₂ esterase gene and the comparison of the predicted amino acid sequence with partial amino acid sequences of xanthine dehydrogenase (XDH) from two insect counterparts, the fruit fly *Drosophila pseudoobscura* (Hughes *et al.*, 1992) and the bluebottle fly *Calliphora vicina* (Houde *et al.*, 1989) (B). The open reading frame is in the opposite direction to the A₂ esterase gene and the predicted amino acid sequence was found to be homologous to the enzyme XDH. Gaps (-) were introduced to maximise the alignment, upper case is used where more than one sequence is the same and an asterisk (*) denotes the same residue in all three sequences.

A

```
attctggaagacactggagaaagcatcaacccggcgatcgatgtcggacagattgagggga 60
  I L E D T G E S I N P A I D V G Q I E G 20

gcgttcgtgatgggaatcggactgtacttcaccgagaacttgatctacagtgacgacaac 120
  A F V M G I G L Y F T E N L I Y S D D N 40

ggacaagttttgacgaatcgaacgtggaactaccatcttccgggagcgaaggatattccg 180
  G Q V L T N R T W N Y H L P G A K D I P 60

gttgatttccgagttaagctgctgcacaacacgttcaacgagaaatttgttctacgctcg 240
  V D F R V K L L H N T F N E K F V L R S 80

aagaccaccggagaaccagcgctgaacatgacggtatcgctgctgttttcgctgagacac 300
  K T T G E P A L N M T V S L L F S L R H 100

gcgctgaactctgctcgcaaggatgctggactttcggatgattgg 345
  A L N S A R K D A G L S D D W 115
```

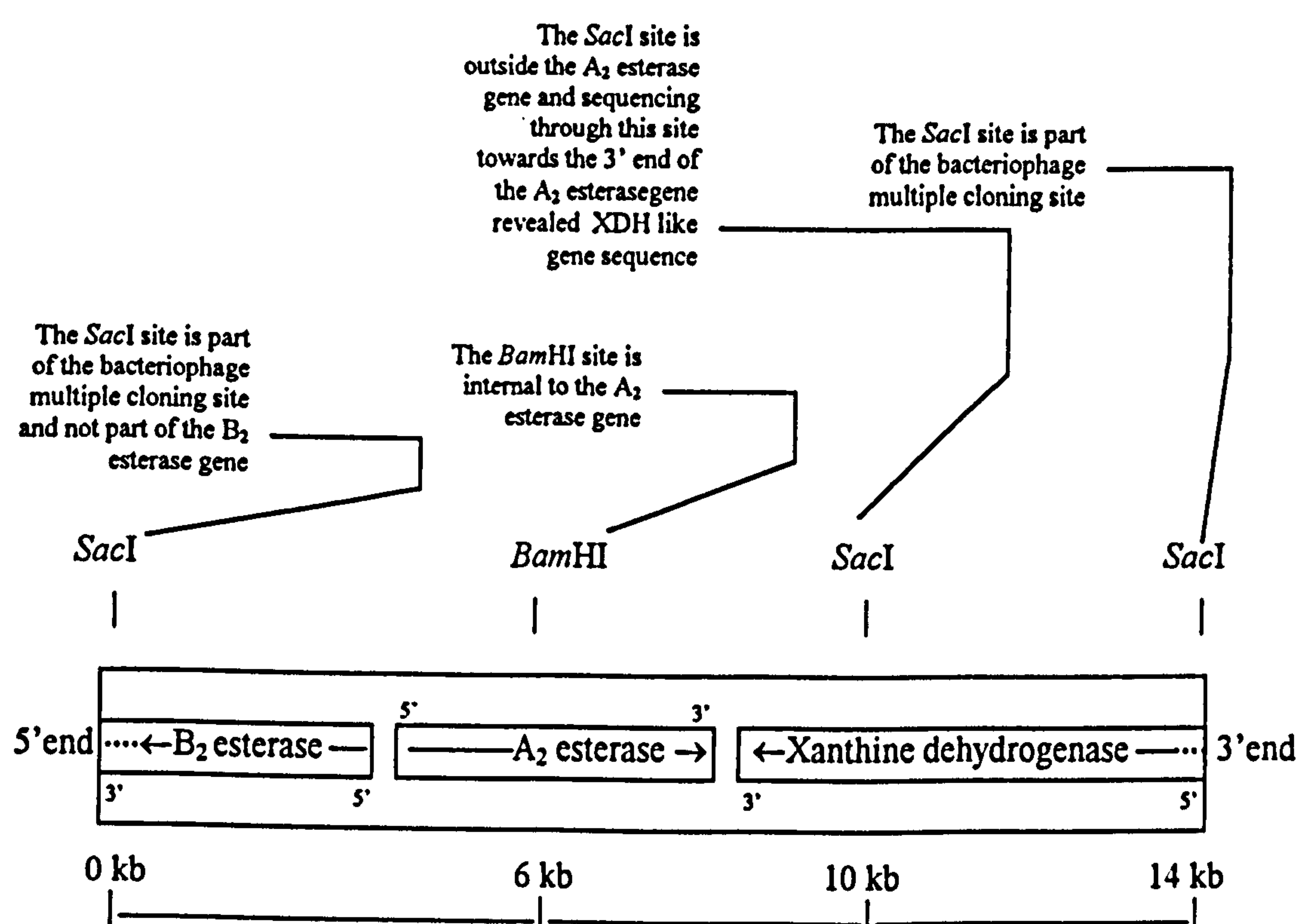
B

```
Cx quinquefasciatus IleDtGeSINPAIDvGQIEGAFvmGiGLyftEnLIYSddn 40
D. pseudoobscura    *VM*I*S*I*****I*****MQ*Y**FTL*ELm**PQ- 39
C. vicina           *VM*I*S*L*****I*****MQ*Y**FTL*EmI**PQ- 39

Cx quinquefasciatus GqvltnrtwnYhLPGakDIPvdFrvkLLhntfNekfVlrS 80
D. pseudoobscura    *mLYSRGPGM*K**~FA***GE*N*sL*TGAP*PRA*YS* 78
C. vicina           *vLYSRGPGM*K**GFA***GE*N*ti*TGAA*PRA*YS* 79

Cx quinquefasciatus KttGEPaLnmtvSllFslrhAlnSARkdaGLSdDw 115
D. pseudoobscura    *AV***P*FIGs*aF*AIKE*Iaa**geq**tg*F 113
C. vicina           *AV***P*FIGc*vF*AIKE*ItS**lmn**se*F 114
```


FIGURE 5.4 A schematic representation of the insert from bacteriophage A2.1 showing the PelRR A₂ esterase gene and the partial length B₂ esterase and suspected XDH genes.



The 14 kb insert was cut out of the bacteriophage DNA by *SacI* (a restriction enzyme site in the multiple cloning site of the bacteriophage arms). The 10 kb *SacI* fragment was cut into a 6 kb and 4 kb fragment with *Bam*HI and these two fragments were subcloned into pBluescript (Stratagene) and partially sequenced. The 6 kb fragment (plasmid pBlue5'AV.A2; METHODS 5.2.1.4) contained the 5' end of the A₂ esterase gene and the 5' end of the B₂ esterase gene. The 4 kb fragment (plasmid pBlue3'AV.A2; METHODS 5.2.1.4) contained the 3' end of the A₂ esterase gene and a partial sequence homologous to the XDH gene. The A₂ and B₂ esterase genes are in a tail to tail arrangement and the A₂ esterase and XDH genes are in a head to head type arrangement.

PCR was successfully used to isolate a partial sequence of the A esterase gene from the MRES strain, which has a unique B₁ esterase gene (CHAPTER 3). Two PCR products were sequenced and were identical. It was assumed that this sequence was that of the amplified A esterase gene present in MRES. The MRES A esterase gene sequence, when compared with PelRR A₂ esterase, covered the latter part of exon 4, intron 4 and the initial part of exon 5, a total of 495 bp. The partial MRES A esterase gene sequence was compared to that of A₂ from PelRR. There were nine nucleotide differences between the two sequences and the comparison between the two is shown in FIGURE 5.5A. There were no nucleotide differences between the two introns (assuming that the intron in the MRES gene is in the same location as that of the PelRR gene). Assuming that the introns were in the same position, the partial amino acid sequences of the two A esterases were also compared and there were two differences, as shown in FIGURE 5.5B.

5.4 CONCLUSIONS

The amplified A₂ esterase gene associated with OP resistant has now been isolated and sequenced from the PelRR strain of *Cx quinquefasciatus*. The A₂ esterase gene, like the B esterase genes code for a protein of 540 amino acids. There are three introns and four exons in the TEM-R B₁ esterase gene, while the PelRR A₂ esterase gene has seven introns and six exons. The position of the three introns in the TEM-R B₁ esterase gene are repeated in PelRR A₂ esterase gene. Introns 1 and 2 are in the same location and intron 3 of the TEM-R B₁ gene aligns with intron 5 of the PelRR A₂ gene. All of the A₂ esterase gene introns are short, ranging from 53 bp (intron 5) to 71 bp (intron 2). Introns 2 and 3 from TEM-R B₁ esterase are also short (52 bp and 61 bp respectively) but intron 1 is

FIGURE 5.5 The nucleotide (A) and predicted amino acid (B) alignment between a partial length A esterase gene from the OP resistant *Culex quinquefasciatus* strain, MRES and the complementary sequences of the A₂ esterase gene from the OP resistant *Culex quinquefasciatus* strain, PelRR. The nucleotide sequence spans intron 4 from the PelRR A₂ esterase gene and this was removed from the MRES sequence (the introns were identical) before the prediction of the amino acid sequence was made. The site of the intron is marked by two vertical red lines. Where the MRES A esterase gene sequence differs from the PelRR A₂ esterase sequence, the two residues are shown in lower case.

A

PelRR	GCCaACTTTGGGGGTGACCCGAAGAACATTACGCTGTTTGGAGAGAGTGC	50
MRES	***t*****	
PelRR	CGGCGGTTGTTCCGTGCACTACCACATGGTGTCGGACCTTTCGCGGGGAT	100
MRES	*****	
PelRR	TGTTTCAGCGtGCGATCGTAATGTCCGGCTGTGTGCTGAACAATTGGTCC	150
MRES	*****c*****a*****	
PelRR	GTGGTTCCACGAAGAAAGTTTAGCGAACGGCTGGCCAAAGCTCTCGGATG	200
MRES	*****	
PelRR	GAACGGGCAGGGTGGTGAGCGAGCTGCTCTGGAGGTGCTCGTGAAGGCGG	250
MRES	*****	
PelRR	ATCCGGAGAGTATCGTTCGtGAGCAGGAAGTTTTGTTGAATGAGAAT	300
MRES	*****a*****	
PelRR	AGAATGATAGCTTGTTAGCTTCAATAGTTGATATCCACTGCATTTAATT	350
MRES	*****	
PelRR	GTAG	400
MRES	****	
PelRR	CCTACATTACAAAaAAaTGCATGATTCCAAAAGATCCTGTAGAGATGTGT	450
MRES	*****g**t*****	
PelRR	CGTGAAGCTTGGAGTAACGAAATCGACATtTTGATCGGCGGAACT	495
MRES	*****c*****	

B

PelRR	AnFGGDPKNITLFGESAGGCSVHYHMSDLSRGLFQRAIVMSGCVLNNWS	50
MRES	*y*****	
PelRR	VVPRRKFSERLAKALGWNQGGERAALEVLVKADPESIVREQEVLNENE	100
MRES	*****	
PelRR	IENRILFSFGPVIEPYITKkCMIPKDPVEMCREAWSNEIDILIGGN	146
MRES	*****n*****	

very long (775 bp). It has already been shown that the A₂ and B₂ esterases have a high percentage similarity (49.2%) and the same number of amino acids. This evidence, along with the similar location of the introns between the two esterases strongly suggests that the two genes arose from a duplication.

In sequencing out from the A₂ esterase gene both 5' and 3', two further open reading frames were encountered. Sequence which had 100% identity to the PelRR B₂ esterase gene was found 5' to the A₂ esterase gene, running 5' to 3'. Assuming that this B₂ esterase gene is functionally active, the two resistance-associated esterase genes are situated in a head to head conformation within the same amplicon and less than 2 kb apart. This would explain why there is complete linkage disequilibrium between the two esterases, as it would be impossible for a recombination event to divide the two esterases in each amplicon. The A₂/B₂ esterase phenotype is found wherever OP resistance occurs in *Cx quinquefasciatus* and this could be due to the fact that the two genes are on the same amplicon and are thus always co-elevated. It is likely that the presence of two resistance-associated genes within a population is more advantageous than one. Therefore, such a mechanism could have been selected over time but given that we now believe the two esterases arose from the duplication of a common ancestral gene, it is possible that they were located next to each other after this initial event. This would be supported if we were able to show that the two genes are next to each other in the PelSS strain. However, analysis of *Eco*RI fragment sizes for the A esterase genes from the PelRR and PelSS strains differ, whereas the B esterase gene fragment sizes do not. The low copy number (probably only one copy) B esterase gene has an *Eco*RI fragment of 3.3 kb in both strains but the low copy number (probably only one copy) A esterase gene *Eco*RI fragment is 7.5 kb in

PelRR and 7.4 kb in PelSS. This could be due to the two A esterase genes being in different locations in comparison to their B esterase genes. The isolation and sequencing of the B or A esterase gene from the PelSS and surrounding sequence will show if the two genes are close neighbours or not. If they are, then it is easy to imagine an amplification of the DNA region surrounding the two genes being the initial step in the resistance-associated mechanism. Further evidence suggests that the two esterases sit together, since co-elevated A and B esterases are also seen in other OP resistant *Culex* species including *Cx tarsalis* and *Cx tritaeniorhynchus* (Prabhaker *et al.*, 1987; Takahashi & Yasutomi, 1987). If the two esterases do not normally sit together, it is difficult to imagine how the same resistance mechanism (co-amplification of A and B esterase genes) could have happened on so many occasions and in different *Cx quinquefasciatus* strains. The fact that the co-elevation of the A and B esterases occurs in different species does imply that the original gene duplication occurred before the speciation of the *Culex* group. Since, it has been shown that the MRES *Cx quinquefasciatus* strain has an amplified A, as well as B esterase, it is possible that the TEM-R strain (and other strains where there is apparently only amplification of a B esterase gene) also has an amplified A esterase. Such findings would add further strength to the argument that the two genes sit together on the chromosome.

At present, the significance of the discovery of a third gene within the A_2/B_2 esterase gene amplicon is unclear. The partial sequence of the XDH gene runs 5' to 3' towards the 3' end of the A_2 esterase gene. This means that the A_2 esterase and XDH genes are in a tail to tail orientation. It is not clear at present, if the XDH gene found in the amplicon is functionally active. The positive bacteriophage clone containing the A_2 esterase gene (bacteriophage A2.1) has an

insert of approximately 16 kb. The first 10 kb of this insert is subcloned into the two plasmids, pBlue5'AV.A2 and pBlue3'AV.A2 (this plasmid contains the 3' end of the XDH gene). The latter 6 kb of insert DNA should hopefully contain the remaining part of the XDH gene, assuming that the gene is full length. Using a 5' part of the XDH gene as a probe on Southern blots of digested PelRR and PelSS genomic DNA would show if the XDH gene was amplified in the PelRR strain and if other similar copies of the gene existed in the PelRR strain. As for the A and B esterase genes, it is likely that an unamplified copy of the gene is present in the resistant (as well as the susceptible) strain. Northern blots of mRNA from the two strains probed with a XDH gene probe would show if there was an increased transcription of the gene. However, XDH mRNA levels might be under tight developmental control in the mosquito and an increased gene copy may not necessarily be associated with an increase in mRNA levels. In *D. melanogaster*, it has been shown that mutants of the *rosy* gene (which is the structural gene for XDH) are highly hypersensitive to oxygen stress (Hilliker *et al.*, 1992) and if XDH is involved in the scavenging of deleterious oxygen radicals, it is likely that its levels would be under a tight control.

The MRES strain of *Cx quinquefasciatus* has an elevated A esterase and the 496 bp PCR genomic DNA product coding for this esterase had a very high identity to the same PelRR gene fragment (98.2% nucleotide identity and 98.7% amino acid identity). In comparison, the amplified B esterase cDNAs from the two strains show slightly less identity (98.0% nucleotide identity and 98.2% amino acid identity). However, only a partial MRES A esterase sequence was available for this comparison and the true identity between the two A esterase genes will transpire when a full sequence comparison is available. A₂ esterase intron 4 from

PelRR is identical to its homologue from MRES. As introns are likely to be under a lesser degree of selection pressure, this suggests that the two amplified A esterases genes are very closely related. In comparison, introns 2 and 3 from TEM-R B₁ esterase are quite different to the comparable introns from PelRR B₂ esterase.

The construction of an MRES genomic library would aid in the isolation of the A and B esterase genes associated with OP resistance. Since a partial MRES A esterase gene sequence has a very high identity with the PelRR A₂ esterase gene, it will be important to see if the two B esterase genes are as equally identical and if the amplified A and B esterase genes are present on the same amplicon. The B esterase gene counterparts may not share a high identity, whereas the A esterase genes may. If this is the case and the two genes sit on the same amplicon, then it would suggest that the two genes were independently brought together onto that amplicon. Otherwise, both genes would share a very high identity to both the PelRR counterparts. Alternatively, the two MRES esterase genes could be contained on separate amplicons. However, as previously discussed, it seems likely that the A and B esterase genes always sit next to each other, whether amplified or not.

The construction of a genomic library from the PelSS strain would also be useful for looking at the genomic arrangement of the A and B esterase genes. If the two genes are located near each other within the genome of a susceptible strain, this would suggest that the co-amplification of the A and B esterases in resistant strains was due to an amplification of the two closely related genes. If however, the two esterase genes are not close to each other (even on separate

chromosomes) then this would suggest that in those resistant strains with amplified A and B esterases that the genes were either amplified separately or by chance, the two genes had been brought together on the same amplicon and were thus always co-amplified.

CHAPTER 6

CHAPTER 6: PELRR A₂ ESTERASE EXPRESSION IN THE BACULOVIRUS SYSTEM

This CHAPTER describes the use of the baculovirus expression system for the expression of *Cx quinquefasciatus* recombinant A₂ esterase from PelRR. As the system is eukaryotic in nature, recombinant eukaryotic proteins are more likely to undergo the correct post-translational modifications that occur *in vivo*. The added advantage to using this system, in this particular case, is that the baculovirus infects an insect cell line and the A₂ esterase is also of insect origin and thus more likely to be expressed exactly as it is *in vivo*.

6.1 INTRODUCTION

The A₂ esterase has been successfully purified and characterised from a number of resistant mosquito strains (Kettermann *et al.*, 1992; 1993; Karunaratne *et al.*, 1995). However, the purification procedure is long and a large amount of starting material is required. The A₂ esterase cDNA has now been isolated (CHAPTER 4) and this opens up the possibility of expressing the recombinant protein. If the protein could be easily expressed and purified, this would be a viable option over purification from whole larvae and would yield enzyme for further kinetic studies as well as for conformation studies.

The baculovirus expression system is beginning to be more widely used and is a versatile high level expression vector of prokaryotic and eukaryotic genes (reviewed by Maeda, 1989; Miller, 1988). The baculovirus used in the expression system is the *Autographa californica* nuclear polyhedrosis virus (AcNPV). Two

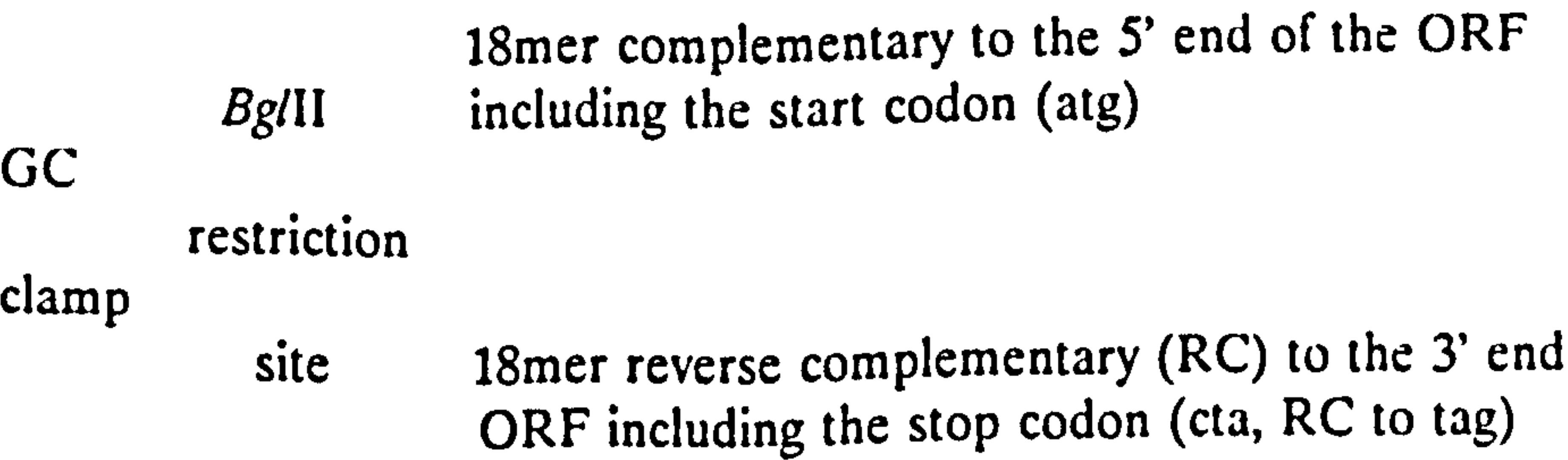
genes are expressed in copious quantities, throughout the very late phase of the viral life cycle. These are the 10 kDa p10 and 29 kDa polyhedrin proteins. Polyhedrin constitutes the major component of the polyhedra and the p10 protein plays a role in polyhedra formation (Vlak *et al.*, 1988; Williams *et al.*, 1989). Since the two proteins are expressed in such large amounts, recombinant proteins expressed under the influence of the promoters of either the polyhedrin or p10 genes can result in very high yields. If the A₂ esterase could be expressed in large amounts in the baculovirus system and purified, then sufficient enzyme could be generated to attempt crystallisation, which will lead to the determination of the esterase's three-dimensional structure.

6.2 METHODS

6.2.1 PCR and subcloning

To isolate a full-length A₂ esterase cDNA from PelRR, two adaptor primers were constructed to the 5' (the start methionine codon, ATG) and 3' (the stop codon, TAG) end of the open reading frame (ORF) of the A₂ esterase cDNA. Each primer contained a GC clamp and a *Bgl*III restriction site at the 5' end. The GC clamp increases the annealing temperature of the primer and the *Bgl*III restriction site allows the PCR product to be easily digested out of any vector it is subcloned into. The two primers were:

For the 5' end: 5'- gcgc - agatct - atggacgtcgaacacccg -3'



For the 3' end: 5'- gcgc - agatct - ctaataaagcttatcttt -3'

The 50 μ l PCR reaction contained 2.5 ng of double stranded PelRR cDNA, 50 ng of each primer, 0.5 mM dNTPs, 2 mM $MgCl_2$ and was buffered in *Taq* DNA polymerase buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100]. The reaction mixture was overlaid with mineral oil and heated in a DNA Thermal Cycler (Hybaid) to 94°C for 3 minutes. The mixture was removed and 2.5 units of *Taq* DNA polymerase (Promega) were added to the reaction which was then heated to 94°C for a further 3 minutes. 40 cycles of amplification were then carried out using a step programme (50°C, 1 minute; 72°C 2 minutes and 94°C, 1 minute). The full length coding region A_2 esterase PCR product was ligated into the pBluescript T-vector (METHODS 2.2.5.4) and after transformation into competent *E. coli* XL1-Blue cells, was cut from the vector with *Bgl*II and ligated into the baculovirus transfer vector pACUW21 (Pharmlngen; Weyer *et al.*, 1990). The PCR product was ligated into the pBluescript initially, in order to obtain sufficient recombinant plasmid DNA for the ligation in to the pACUW21 vector. Recombinant plasmid DNA was isolated and the A_2 esterase ORF was cut from the plasmid with *Bgl*II. The A_2 esterase ORF was then ligated into the unique *Bgl*II site in the pACUW21 vector. pACUW21 has a copy of the p10 gene promoter and SV40 transcription termination sequences inserted upstream of the complete polyhedrin gene. The A_2 esterase ORF was inserted into a unique *Bgl*II site downstream of this p10 promoter and upstream of the SV40 termination sequence. Since pACUW21 contains the polyhedrin gene downstream of the p10 promoter and unique *Bgl*II site, recombinant viruses will be able to form polyhedra. The recombinant pACUW21 vector (pACA2) was digested with restriction enzymes unique to the pACUW21 vector and the A_2 esterase ORF to ensure the ORF had been orientated correctly.

6.2.2 Cotransfection of the recombinant plasmid (pACA2) and the baculoviral DNA (AcNPV) into the fall armyworm *Spodoptera frugiperda* insect host cells

Spodoptera frugiperda (clone IPLB-Sf-21, known as Sf21, Vaughn *et al.*, 1977) cells were used for the cotransfection and 10^6 cells were plated in 35 mm petri dishes in 2 mls of ExCell 401 growth medium (JRH Biosciences) + PS (1% v/v 10000 units/ml penicillin and 10000 μ g/ml streptomycin, GibcoBRL) and allowed to settle for 45 minutes. This number of cells allows the propagation of the virus and plaque identification. In a 15 ml polystyrene tube, 0.5 μ g of pACA2 was mixed with 50 ng of BacPAK6 viral DNA (Clontech) in a total volume of 12 μ l. The BacPAK6 genomic DNA had been digested with *Bsu*361 to remove part of an essential viral gene (Kitts & Possee, 1993). Only recombination with the pACA2 plasmid replaces the essential gene sequences and therefore nearly 100% of viruses recovered from cotransfection will contain the recombinant full length ORF (of the A₂ esterase). 12 μ l of a solution containing 8 μ l of Lipofectin reagent (GibcoBRL) and 4 μ l of deionised water were then added and the contents of the tube gently mixed and left at room temperature for 15 minutes. Lipofectin reagent and DNA form a lipid-nucleic acid complex which, when applied to cultured cells, fuse with the plasma membrane and transfer the nucleic acids into the cells. The cells were washed twice with ExCell 401/PS (2 mls per wash) and then 1 ml of ExCell 401/PS was added to the cells along with the 24 μ l cotransfection mixture and mixed gently. After 5 hours another 1 ml of ExCell 401/PS was added and the dishes were incubated at 28°C for four days.

6.2.3 Plaque assay

The plaque assay (an adaptation of Brown & Faulkner, 1977) was carried out 48 hours after infection (if the plaque assay is negative, it is repeated 96 hours after

infection) of the Sf21 cells with the virus. Medium surrounding the cells (500 μ l) was removed and if the transfection was successful, this would contain budded viruses, which are able to infect other Sf21 cells. Petri dishes were seeded with 10^6 Sf21 cells in 2 mls of ExCell 401/PS/FCS (ExCell 401/PS with 3% v/v foetal calf serum, Sigma) and left for 45 minutes to settle. The medium was removed and 100 μ l of a 10^0 and a 10^{-1} dilution of the cotransfection medium were inoculated in duplicate, onto separate petri dishes. After an hour (in which time the budded viruses will have infected the Sf21 cells), the inoculum was removed and the cells were overlaid with 2 mls of a 1:1 mixture of ExCell 401/PS/FCS and 3% (w/v) low melting point agarose at 37°C. After the agarose had set, the dish was overlaid with 1 ml of ExCell 401/PS/FCS and incubated for 72 hours at 28°C. The agarose stabilises the cells allowing the viral plaques to be identified. To each dish, 1 ml of a 1:20 dilution of 0.5% (w/v) neutral red (Sigma) in PBS, was added and left for 2 hours. The neutral red and medium were then removed and the plates inverted and incubated at 4°C overnight. Neutral red stains living cells and clear plaques were seen on the plates due to the death of the cells infected by virus. Plaques were examined under a phase contrast microscope to ensure there was polyhedra formation. Polyhedra positive plaques were removed with a sterile glass pipette into 500 μ l of ExCell 401/PS/FCS and stored at 4°C overnight. Further plaque assays were performed on the positive plaques until all plaques were polyhedra positive.

6.2.4 Amplification of the budded recombinant baculovirus (AcNPVA2)

The pure plaque stock was used to amplify AcNPVA2. A 35 mm petri dish was seeded with 0.5×10^6 Sf21 cells in 2 mls of ExCell 401/PS/FCS. After 45 minutes, the medium was removed and 100 μ l (20%) of the pure AcNPVA2 plaque stock

was added. This was allowed to infect for 1 hour, the inoculum removed and 2 mls of ExCell 401/PS/FCS were added and the plate incubated at 27°C for 5 days. In this time, the AcNPVA2 budded viruses infect the cells, the virus reproduces and more budded viruses are released which further infect more cells. Polyhedra also form in the cells, many of which die. The medium and cells were removed and centrifuged at 4°C and 1000g for 10 minutes, leaving a debris of cells and a supernatant containing a stock of budded AcNPVA2 viruses. The cells and supernatant were stored at -20°C. Two large tissue culture flasks (75 cm² surface area) were seeded with 1×10^7 Sf21 cells in 10 mls of ExCell 410/PS/FCS and left for 45 minutes. The medium was removed and 300 μ l (15%) of the amplified AcNPVA2 budded virus inoculum was added to each and left for 1 hour. Twenty five millilitres of ExCell 410/PS/FCS was added to each flask and the flasks incubated at 27°C for 5 days. As before, the cells and medium were removed, centrifuged at 4°C and 1000g for 10 minutes and the amplified stock of AcNPVA2 budded virus were stored in the dark at 4°C. This stock was used to produce a polyhedra stock for bioassays.

6.2.5 Assay of esterase activity

To ensure that the recombinant protein had esterase activity, an esterase assay was carried out on the cells and the supernatant from the initial amplification of the positive plaque (carried out in a 35 mm petri dish, METHODS 6.2.4). The pelleted cells (approximately 5×10^5) were resuspended in 100 μ l of ice cold 0.1 M Tris-HCl (pH 7.5) and lysed by three freeze/thaw cycles using an ethanol/dry ice bath. After centrifugation at 10000g for 5 minutes, 20 μ l of the cell lysate (or 20 μ l of the medium supernatant) were assayed in triplicate for esterase activity. A further 20 μ l of cell lysate was used for native polyacrylamide gel

electrophoresis (native PAGE) (METHODS 6.2.6). Fast Blue B assay buffer [62.5 mg of Fast Blue in 100 ml of 0.1 M Tris-HCl (pH 7.5)] (278 μ l) was added to 20 μ l of a suitably diluted cell lysate supernatant (or 20 μ l of the medium supernatant) in a microtitre plate. Two microlitres of the substrate α -naphthyl acetate [13.97 mg in 1 ml of 50% (v/v) acetone] were added and after an initial mix, the increase in absorbance was measured at 450 nm for 2 minutes (UV-Max). The esterase activity was measured as μ moles of α -naphthol produced/minute/lysate from 10^6 cells. Activity was calculated from a standard curve of the product, α -naphthol.

6.2.6 Native polyacrylamide gel electrophoresis (Native PAGE)

To establish that esterase activity was present in the AcNPVA2 baculovirus, native PAGE was carried out. Cell lysate was used from the initial plaque amplification (METHODS 6.2.4). As controls, cell lysate samples of SF21 cells uninfected and infected with wild-type baculovirus (AcNPV) were used. Electrophoresis was performed using 7.5% acrylamide gels in Tris-borate/EDTA buffer (pH 8.0) [0.1 M Tris-HCl, 2.5 mM EDTA, 40 mM boric acid] by the method of Davis (1964). The gels were either stained for protein with Coomassie Blue R250 or for esterase activity with 0.04% (w/v) α -naphthyl acetate and 0.1% (w/v) Fast Blue B in 100 mM phosphate buffer (pH 7.4) and then fixed in 10% acetic acid before drying onto Whatman 3MM paper.

6.2.7 Amplification of recombinant AcNPVA2 polyhedra

Cabbage looper *Trichoplusia ni* (*T. ni*) cells (clone BTI-TN-5B1-4, commonly known as High Five) in spinner culture were used for polyhedra preparation as the cells are bigger and more polyhedra can form in each cell. Using the

amplified AcNPVA2 budded recombinant virus stock (2.4×10^8 budded viruses/ml), 1 ml were added to a spinner flask containing 50 ml of *T. ni* cells at a concentration of 10^6 cells/ml in ExCell 401/PS. Since there are approximately 5 times as many budded viruses as there are cells, this ensures that virtually all the cells are infected with virus. The cells were left for an hour and then a further 50 mls of medium was added and the flask left to spin at 130 rpm for 7 days. The culture, which then contained a very large number of polyhedra, was centrifuged at 1000g for 10 minutes to remove cellular debris. If there had been extensive cell lysis the polyhedra would be in the supernatant. If not, the polyhedra would still be in the cells. If this was the case the cells were resuspended in 10 mls of 0.05% (w/v) SDS (if necessary increasing to 0.5%) to lyse the cells and release the polyhedra. The cell debris was then removed as before and the two supernatants were centrifuged at 5000g at 4°C for 20 minutes to pellet the AcNPVA2. The pelleted AcNPVA2 polyhedra were resuspended in an equal volume of 0.5 M NaCl, pelleted as before and finally resuspended in 1 ml of deionised water and stored at -20°C or at 4°C with the addition of 0.02% (w/v) sodium azide.

6.2.8 A₂ esterase expression from the AcNPVA2 recombinant baculovirus in insect cell lines

The baculovirus expression system is suited for the production of large amounts of recombinant protein which can then be further purified, characterised and even crystallised. Baculovirus expression is commonly undertaken in either Sf21 cells or *T. ni* cells. Expression of the recombinant protein can be different in the two cell lines. Performing a time course study of protein expression with both lines and assessing esterase activity, will show which cell line produces the most recombinant protein. This cell line can then be used for large scale protein

production. Using the amplified AcNPVA2 budded recombinant virus stock (2.4×10^8 /ml), 1 ml was added to a spinner flask containing 50 ml of *T. ni* cells at a concentration of 10^6 cells/ml in ExCell 401/PS. A similar time course was undertaken using 500 μ l of the virus stock and 25 ml of Sf21 cells at a concentration of 10^6 cells/ml in ExCell 401/PS/FCS. The virus to cell ratio of about 5 ensures that virtually all the cells are infected with a virus. The cells were left for an hour and then a further 50 (25) mls of medium was added and the flasks left to spin at 130 rpm. At 17, 26, 43, 48, 66, 72 and 88 hours post infection, 1 ml of cells were removed from each flask, frozen and assayed for esterase activity (METHODS 6.2.7). Cells infected with the wild-type baculovirus (AcNPV) were used as a negative control for the experiment.

6.2.9 Bioassay of the expressed A₂ esterase

Recombinant baculoviruses expressing a variety of proteins have been used to try and improve the insecticidal properties of baculoviruses by decreasing the time taken to kill the insect. Initial studies on baculoviruses expressing recombinant juvenile hormone esterase (JHE) suggest that such bio-insecticides could decrease the kill time of the insect (Hammock *et al.*, 1990; Bonning *et al.*, 1992). JHE and A₂ esterase are carboxylesterases and the possibility exists that the A₂ esterase could itself have an effect on the kill time of insects (increasing or decreasing) or could have no effect at all. If the latter is true, then a recombinant baculovirus expressing the A₂ esterase would act as a good negative control when comparing the kill time of recombinant baculoviruses expressing different carboxylesterases (JHE for example).

To determine the insecticidal properties of the AcNPVA2 baculovirus, newly hatched *T.ni* larvae were used. *T. ni* were maintained in the laboratory on a semi-synthetic diet (Hunter *et al.*, 1984). To determine the LT_{50} of the AcNPVA2 virus when compared to the wild-type virus (AcNPV), the larvae were infected using the droplet feeding method at 100 pib (polyhedra inclusion bodies)/ μ l (Hughes *et al.*, 1986). The larvae were placed in the middle of a petri dish and drops of the virus were applied in a circles around the larvae. As the larvae moved towards the outside of the dish they fed on the virus. Each fed larva (30 for each virus) was transferred to an individual tub of artificial diet and mortality was monitored daily at 6 or 8 hour intervals according to the mortality rate. LT_{50} values were determined with the Visit Program (Boyce Thompson Institute, Ithaca, New York, 1990).

6.3 RESULTS

After the initial cotransfection and subsequent plaque assay, a single polyhedra positive plaque was picked and assayed again. This second assay gave plaques which were all polyhedra positive and it was hoped that this was a recombinant baculovirus containing the ORF of PelRR esterase A₂. The budded baculovirus was successfully amplified and the esterase assay (METHODS 6.2.5) from the initial amplified 35 mm petri dish (METHODS 6.2.4) suggested that a functional esterase had been expressed within the Sf21 cells. No esterase activity was found in the medium of either cells infected with AcNPVA2 recombinant virus or cells infected with AcNPV wild-type virus. A small degree of activity was detected in AcNPV infected cell lysate (26.6 μ moles of α -naphthol produced/minute/lysate from 10^6 cells). The AcNPVA2 infected cell lysate however, had a very high esterase activity (428 μ moles of α -naphthol produced/minute/lysate from 10^6

cells). A native PAGE gel was run with freeze-thawed Sf21 cells expressing the A₂ esterase, as well as controls with uninfected cells and cells expressing the AcNPV. The gel was stained for both protein and esterase activity and the results, shown in FIGURE 6.1, suggested that a substantial amount of protein, able to hydrolyse α -naphthyl acetate, was being expressed only in the Sf21 cells containing the AcNPVA2. The recombinant A₂ esterase was expressed in spinner culture in both Sf21 and *T. ni* cells, in order to assess which cell line gave the higher expression levels. The results of the time course experiment are shown in TABLE 6.1 and maximum expression of the protein was achieved after 72 hours in *T.ni* cells. Cells infected with the AcNPV were also assayed at the same time and assayed for esterase activity. This activity was subtracted from the AcNPVA2 recombinant activity to give a true measure of esterase activity due to the recombinant A₂ esterase. The AcNPVA2 was also used in LT₅₀ bioassays to determine if the presence of the esterase would make a marked difference on the kill time when compared to the AcNPV. The results from this experiment were 119 hours (between 110 and 128 hours at a 95% confidence limit) for the AcNPVA2 and 117 hours (between 113 and 121 hours at a 95% confidence limit) for the AcNPV, suggesting that the over-expression of the esterase in the *T. ni* larvae has no deleterious effect above that of the virus itself.

6.4 CONCLUSIONS

The results suggest that a functional recombinant A₂ esterase from the PelRR OP resistant strain of *Cx quinquefasciatus* has been successfully expressed in the baculovirus system. An increased level of esterase activity was seen in freeze-thawed Sf21 cells and in a native PAGE gel, where staining showed the existence of a highly expressed protein.

FIGURE 6.1 Native PAGE gel of lysates of Sf21 cells. The cells were either uninfected (**Lane 1**), infected with AcNPVA2 recombinant baculovirus expressing esterase A₂ from the OP resistant *Culex quinquefasciatus* strain, PelRR (**Lane 2**) or infected with AcNPV wild-type baculovirus (**Lane 3**). Each lane contains protein from approximately 0.1 x 10⁶ cells. The same samples were run twice and stained for protein (**A**) and for esterase activity (**B**) (**METHODS 6.2.5**).

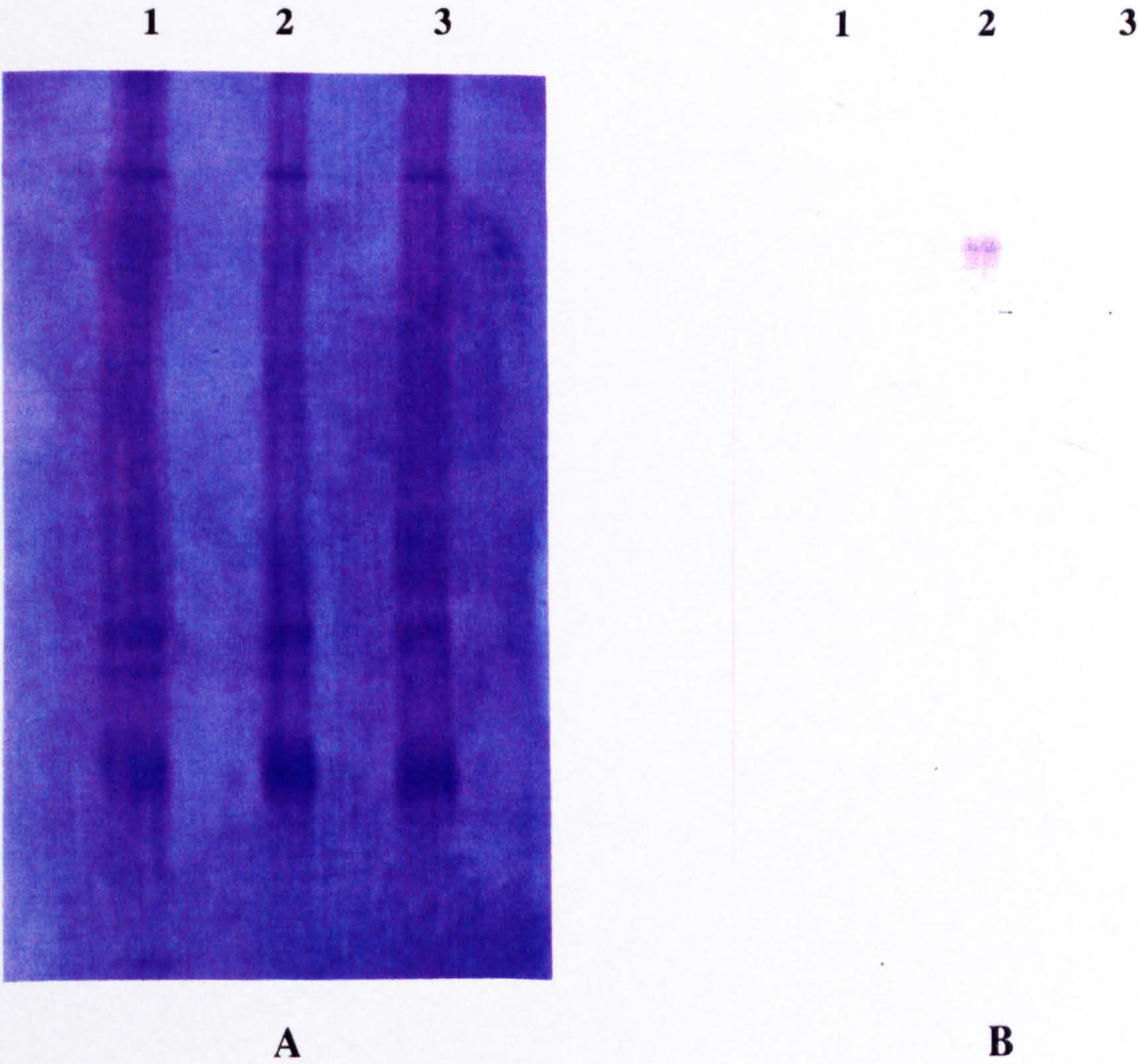


TABLE 6.1 The esterase activity of spinner culture insect cells infected with the AcNPVA2 recombinant baculovirus expressing a functional A₂ esterase from the *Culex quinquefasciatus* OP resistant strain, PelRR.

Hours post infection	Esterase activity (μmoles α-naphthol produced/minute/lysate from 10 ⁶ cells)	
	<i>Trichoplusia ni</i>	<i>Spodoptera frugiperda</i>
17	0.0	0.0
26	0.0	0.0
43	488.0	0.0
48	520.0	127.0
66	524.0	89.0
72	878.0	189.0
88	588.0	452.0

2.4 x 10⁸ (1.2 x 10⁸) budded viruses were added to spinner flasks containing 50 (25) mls of *T. ni* (Sf21) cells at a concentration of 10⁶ cells/ml in ExCell 401/PS (ExCell 401/PS/FCS). The cells were left for an hour and then a further 50 (25) mls of medium was added and the flasks left to spin at 130 rpm. Cells were removed periodically, freeze-thawed and assayed for esterase activity.

The kinetic characterisation of purified A₂ esterases from a number of OP resistant *Cx quinquefasciatus* strains, with different insecticides, has suggested there are differences in the A₂ esterase between the strains (Karunaratne *et al.*, 1993c; Kettermann *et al.*, 1993). N-terminal and internal amino acid sequencing of these esterases has to date been unsuccessful, suggesting that the protein may not be pure (although it appears to be pure on PAGE) (Karunaratne, 1994). Hence, purification of the A₂ esterase from insect strains may be resulting in mixtures of very similar proteins. The make up of these mixtures could explain why kinetic differences are seen between the different A₂ esterases. *EcoRI* fragment analysis of the *Cx quinquefasciatus* PelRR strain A₂ esterase gene has shown that as well as a highly amplified A₂ esterase gene, a low copy number (probably only one) allele is also present (CHAPTER 4). The restriction enzyme fragment sizes of amplified A₂ esterase genes from four different *Cx quinquefasciatus* strains are identical, although the amplification level of the gene varies between strains (S.H.P.P. Karunaratne, personal communication). This suggests that all the A₂ esterases are identical. In the purification of the various A₂ esterases, the esterase expressed at normal levels could also have been co-purified. The percentage contribution of this esterase would be different in each strain, due to the varying levels of amplification of the A₂ esterase. Therefore, the presence of the low copy number (probably only one) esterase gene could explain why the A₂ esterases from the different strains seem kinetically distinct. Alternatively, there may be nucleotide differences between the A₂ esterase genes from the different strains or more than one resistance-associated allele. These could lead to amino acid and kinetic differences between the proteins. By using the baculovirus expression system to purify protein, there is a guarantee of a pure enzyme, the product of a single gene. If A₂ esterases from the other strains were expressed in the

baculovirus system and behaved identically to the PelRR A₂ esterase, this would show that other A esterases were present in the A₂ esterase purified from larvae. If there were differences between the A₂ esterases, then sequencing the genes would reveal the nucleotide differences and subsequent amino acid differences. Since the restriction enzyme patterns for the A₂ esterase genes are identical between the strains, it is likely that nucleotide differences between the genes would have occurred recently, after the A₂ esterase amplification event. It is possible that nucleotide substitutions have occurred in one (or a minority) of the many amplified genes and this in turn would lead to the low level expression of a unique A₂ esterase. The presence of these altered alleles could cause the kinetic differences seen between the purified A₂ esterases. Their presence would be difficult to ascertain, since the A₂ esterase gene is highly amplified. Attempts to find a nucleotide substitution in just one amplified gene by classical molecular biological methods would be far too time consuming. This would involve either the sequencing of multiple genes from genomic libraries or multiple cDNAs from PCRs of the A₂ esterase ORF. A possible solution would be to look for single stranded chain polymorphism (SSCP) in the A₂ esterase gene population. However, SSCP is normally undertaken on small fragments of DNA (around 200 bp) and on unamplified genes and the need to cover the whole A₂ esterase gene and the presence of amplified gene copies could lead to severe difficulties in finding polymorphisms between the amplified genes.

The amino acid sequence of PelRR esterase A₂ does not contain an N-terminal signal sequence or a C-terminal signal for ER retention (although there is no logical reason for the presence of such a C-terminal signal, as the N-terminal signal is not present). The lack of an N-terminal signal implies that the protein

remains cytoplasmic and this is in agreement with esterase activity of the recombinant baculovirus only being found within the cell and not in the medium in which the cells were cultured. Only proteins that are directed through the ER can be glycosylated and therefore, the cytosolic PelRR A₂ esterase is unlikely to undergo any major post-translational modifications. For this reason, it may be easy to express the A₂ esterase in a prokaryotic expression system. This method would be cheaper and easier to utilise than the baculovirus system and could also save time. The carboxylesterase JHE from the major insect pest *H. virescens* has been expressed in the baculovirus system and the esterase (which has an N-terminal signal amino acid sequence) is exported into the medium of the cell culture (Hammock *et al.*, 1990). To date, all the amino-acid sequences for carboxylesterases have an N-terminal signal sequence which directs the mature protein to leave the cell. Most mammalian liver carboxylesterases however, remain in the lumen of the ER due to a C-terminal retention signal (Pelham, 1990; Medda & Proia, 1992). The only esterases not in some way exported (due to the absence of N-terminal sequences) are the *Cx quinquefasciatus* OP resistance-associated A and B esterases. The A and B esterases from *Cx quinquefasciatus* and the E4 esterases from *M. persicae* all sequester the OP insecticides (Devonshire, 1977; Devonshire & Moores, 1982; Ketterman *et al.*, 1992; Cuany *et al.*, 1993; Jayawardena *et al.*, 1994). The E4 esterases have N-terminal signal sequences but no C-terminal retention sequences, so are exported from the cell. There has to be therefore, physiological differences between the two insect sequestration systems since A and B esterases are internal to the cell and E4 esterases are exported. Localisation studies need to be performed on both insect systems to determine how the expression differences are associated with the sequestration of the OPs.

Using spinner cell culture with *T. ni* cells, the recombinant A₂ esterase protein can be produced in sufficient quantities for purification after about 72 hours post infection, when esterase activity reaches its peak. After 88 hours the esterase activity in the Sf21 spinner culture was still rising. Since no more time points were assayed, the maximum esterase activity time could not be elucidated. However, expression typically peaks between 72 and 120 hours and it is unlikely that expression in the Sf21 cells would have exceeded that of the *T.ni* cells within 120 hours, as expression in the *T. ni* cells was twice that of Sf21 (when comparing the maximum assayed activity of the time points). An A₂ esterase purification strategy has already been devised for mosquito larvae (Kettermann *et al.*, 1992). This strategy could be used for the purification of recombinant A₂ esterase. Since the A₂ esterase is over-expressed in the *T. ni* cells, purification should be easier due to the majority of protein being the A₂ esterase and there being no substantial amount of other esterases in the cells. Therefore, the time consuming purification procedure can hopefully be shortened, enabling large amounts of protein to be quickly purified and subsequently characterised. Once pure, kinetic characterisation of the recombinant A₂ esterase with a range of substrates and inhibitors will confirm if it is identical to the A₂ esterase purified from the PelRR strain. The isolation of further A esterase amino acid sequences from OP resistant and susceptible strains will hopefully point to those residues necessary for the increased binding of OPs seen in the resistance-associated esterase compared to the esterases from susceptible strains. These residues could then be mutated in the recombinant A₂ esterase cDNA and the mutant recombinant protein could be expressed, purified and characterised to confirm those residues essential for OP binding. An increased knowledge of the way the enzyme operates will hopefully lead to the synthesis of novel OPs which can interact with AChE but not with the

amplified esterases. In the field, however, the altered AChE mechanism of resistance is becoming more common. Thus, research into new generation insecticides will have to take this into account and the insecticide must powerfully inhibit AChE, altered AChE and not be sequestered by the amplified esterases. However, since the A and B esterases are closely related to AChE and the reaction of the insecticides with the esterases are homologous, synthesising new insecticides may not be too difficult. The insecticide would have to specifically target the AChE while reducing the A and B esterase binding. The three-dimensional structure of *T. californica* AChE is known (Sussman *et al.*, 1991), knowledge of the A₂ esterase three-dimensional structure would point to the differences between the AChE and A₂ esterase active sites and assist in the designing of new insecticides.

The LT₅₀ of the A₂ esterase recombinant baculovirus on *T. ni* larvae was no different to that of the wild-type virus. Since the A₂ esterase is highly amplified in mosquito larvae, it is not surprising that highly amplified A₂ esterase does not have a deleterious effect on the mortality of *T. ni* larvae. The carboxylesterase JHE has been expressed in the baculovirus system with the aim of creating a recombinant baculovirus insecticide (Hammock *et al.*, 1990; Bonning *et al.*, 1992). JHE hydrolyses juvenile hormone (JH) (Hanzlik & Hammock, 1988) and the absence of JH results in loss of larval commitment of the tissues (Riddiford, 1980; Sehna, 1981). Thus, it was hoped that the release of JHE *via* the baculovirus would halt the feeding of insect pest larvae and induce pupation. However, expressing recombinant JHE in the baculovirus system was not as fatal as was hoped when fed to insect pest larvae (Bonning *et al.*, 1992). Therefore it was hoped that by mutating the JHE gene, a more effective baculoviral larvicide could

be created. In experimenting with recombinant JHE containing baculoviruses, the A_2 esterase recombinant baculovirus would act as an excellent negative control since the expression of this carboxylesterase does not have an adverse effect on larvae, whereas the recombinant JHE carboxylesterase would hopefully have an effect.

CHAPTER 7

CHAPTER 7: GENERAL DISCUSSION

7.1 CARBOXYLESTERASE STRUCTURE AND THE ROLE OF ESTERASES IN INSECTICIDE RESISTANCE

The target site for both the organophosphates (OPs) and the carbamates is the carboxylesterase, acetylcholinesterase (AChE). In *Cx quinquefasciatus*, the major OP resistance mechanism is over-production of non-specific carboxylesterases which sequester OPs (INTRODUCTION 1.3.1.1,1.5.3). The elevated A_2/B_2 esterase phenotype is the most common and the present study has shown that the elevation of A_2 esterase is due to gene amplification and has confirmed that the B_2 esterase gene is also amplified. The rapid sequestration of the insecticide by the esterases, mirrors the effect the insecticide has on its target. In a similar way, the AChE sequesters the OP and very slowly turns it over. In resistant insects, the sequestration of the OP by AChE (which can cause death due to the inability of the insect to hydrolyse acetylcholine) is prevented due to the presence of the elevated A and/or B esterases sequestering the insecticide before it reaches AChE. AChE and the A_2/B_2 esterases presumably have similar structures as they react similarly with OPs.

X-ray structures have recently been determined for both *T. californica* AChE (Sussman *et al.*, 1991) and *G. candidum* lipase (Schrag & Cygler, 1993). The carboxylesterases and lipases have been shown to belong to the same enzyme family due to their strikingly similar three-dimensional structures (Cygler *et al.*, 1993). Most carboxylesterases have some degree of lipase activity and both *Cx quinquefasciatus* A_2 and B_2 esterases are able to hydrolyse medium chain length

mono- and di-acylglycerols (Kettermann *et al.*, 1992; Jayawardena, 1992). The three-dimensional structures of AChE and lipase were super-imposed on each other and this enabled 29 other related hydrolytic proteins, including lipases, carboxylesterases, cholinesterases (acetyl- and butyryl-) and cholesterol esterases to be aligned with the two known X-ray structures (Cygler *et al.*, 1993). This alignment showed that 24 residues were invariant in all the enzymes [apart from *H. virescens* JHE (Hanzlik *et al.*, 1989) and the OP resistance linked B₁ esterase from the *Cx quinquefasciatus* strain, TEM-R (Mouches *et al.*, 1990)] and an additional 49 residues were well conserved. The invariant residues included the active site triad, disulphide bridges, salt bridges and residues in the core of the proteins. The common residue in TEM-R B₁ esterase and *H. virescens* JHE not conserved is a disulphide bridge-associated cysteine and a second disulphide bridge-associated cysteine conserved in virtually all the sequences is not present in the TEM-R B₁ esterase. When the A₂ and B₂ esterase amino acid sequences were superimposed on this alignment, the two highly conserved cysteines were not present. The role and importance of the disulphide bridges involving these residues were not apparent from the X-ray structures of the *T. californica* AChE and *G. candidum* lipase alone but it was suggested that both bridges may play a role in substrate binding and/or recognition (Cygler *et al.*, 1993). The isolation of further insect carboxylesterase amino acid sequences may show the absence of the two disulphide bridges, although the two bridges are present in the OP resistance-associated elevated esterase, E4, from *M. persicae* (Field *et al.*, 1993) and the isolated *D. melanogaster* esterases (Oakeshott *et al.*, 1987; Brady *et al.*, 1990; Collet *et al.*, 1990). It seems more likely therefore, that the bridges have been lost in *Cx quinquefasciatus*, as they are retained in virtually all other esterases. A conserved alanine residue in all the aligned esterase sequences is a valine in the

PelRR A₂ esterase (Val³⁷). The reason for the conservation of this alanine in all the other esterases is not clear. However, both alanine and valine have saturated hydrocarbon, hydrophobic side-chains [the alanine side-chain is CH₃, whereas the valine side-chain is CH-(CH₃)₂] and therefore would effect the structure of the esterase in a similar way.

An alignment of residues around the active site triad residues of insect carboxylesterases and all known AChE amino acid sequences is shown in TABLE 7.1. Nine of the residues twenty-four around the active site serine are conserved in all the sequences and eight of these are conserved in all known carboxylesterase sequences (although the conserved alanine residue, directly proceeding the active site serine residue is present in all the sequences shown, it is not conserved in other carboxylesterases). A further eight residues were conserved in the majority of sequences, signifying the importance of the residues in this active region in maintaining the structure and function of the active site serine. The phenylalanine residue, three residues before the active site serine, is conserved in all the AChE's (it is also present in PelRR A₂ esterase), suggesting that it might be essential for AChE activity. The conservation of this phenylalanine residue in A₂ esterase and AChEs could explain why A₂ (unlike B₂ esterase and all other non-AChE carboxylesterases) is inhibited by very low concentrations of the AChE inhibitor eserine (95.1% inhibition at a concentration of 0.01 mM) (Ketterman *et al.*, 1992). The phenylalanine residue could be directly involved in the binding of eserine and its subsequent inhibitory effects. Around the active site serine, MRES B₁ esterase differs from the other *Cx quinquefasciatus* B esterases (Ala→Gly) as does TEM-R B₁ esterase (Val→Ala). The changes are minor (both residues in each case having small hydrophobic,

TABLE 7.1 Identities between amino acid sequences surrounding the three active site residues [serine, histidine and acid (either glutamate or aspartate)] of the insect carboxylesterases (including the *Culex quinquefasciatus* A and B esterases) and acetylcholinesterases (AChEs). The AChEs were included, since they are the target for organophosphate and carbamate insecticides. Amino acids common to all 16 sequences are shown in bold type. If an amino acid residue makes up the majority at any position it is shown in upper case. The three active site residues are underlined.

1. present study; 2. Mouches *et al.* (1990); 3. Oakeshott *et al.* (1987); 4. Collet *et al.* (1990); 5. Field *et al.* (1993); 6. Hanzlik *et al.* (1989); 7. Hall & Malcolm (1991); 8. Hall & Spierer (1986); 9. Schumacher *et al.* (1986); 10. Sikorav *et al.* (1987); 11. Doctor *et al.* (1990); 12. Soreq *et al.* (1990); 13. Rachinsky *et al.* (1990).

Esterase	Amino acid sequences surrounding the three active site triad residues			Reference
	serine	histidine	acid	

Cx quinquefasciatus
A and B esterases:

PeIRR A ₂	NvAnFGGDPkniTLFGESAGgCSV	vrGtaHaDdlSYiFknvf	iGgnseEGlfcLNgiken	1
PeIRR B ₂	NIAAFGGDPkrVTLvGhSAGAAASV	lrGtaHaDElSYlFsnft	iGgtseEGl1lLqkiklq	1
TEM-R B ₁	NIAAFGGDPkrVTLaGhSAGAAASV	lrGtaHaDElSYlFsnft	iGgtseEGl1lLqkiklh	2
MRES B ₁	NIgAFGGDPkrVTLvGhSAGAAASV	lrGtaHaDElSYlFskft	iGgtseEGl1lLqkiklq	1
PeISS B	NIAAFGGDPkrVTLvGhSAGAAASV	lrGtaHaDElFYlFsnft	iGgtseEGl1lLqkiklq	1

Insect carboxylesterases:

<i>Drosophila</i> esterase P	NIAhFGGmPdniVLiGhSAGgASa	hfGtvHgDdfliFntaa	vtyttedGgynaqaqler	3
<i>Drosophila</i> esterase 6	NIAhFGGmPqnlLVghSAGgASV	dfGtvHgDdyfliFenfV	vsyvtedGgynaallke	4
<i>Myzus</i> esterase E4	NIvAFGGDPkrVTitGmSAGASSV	gssptHgDetsYvLkmdg	isiaqdEGliFstflgle	5
<i>Heliothis</i> JH esterase	NaknFGGDPsditiagqSAGASaa	heGvgHiiedltYvFkvns	iGftssEcetFrnrllnf	6

Acetylcholinesterases:

<i>Anopheles stephensi</i>	NakAFGGDPdliTLFGESAGgssV	wmGvlHgDEveYiFgqpm	vGsnrdEGtyFLlydfid	7
<i>Drosophila</i>	NahAFGGnPewmTLFGESAGssSV	wmGvlHgDEieYfFgqpl	mGnvrdEGtyFLlydfid	8
<i>Torpedo californica</i>	NIqfFGGDPktVTiFGESAGgASV	wmGviHgyEiefvFglpl	lGvnkdEGsfFLlygapg	9
<i>Torpedo mamarota</i>	NIqfFGGDPktVTLFGESAGgASV	wmGviHgyEiefvFglpl	lGvnkdEGsfFLlygapg	10
Fetal bovine serum	NvAAFGGDPTsVTLFGESAGAAASV	wmGvpPHgyEiefiFglpl	vGvvkdEGsyFLvygapg	11
Human	NvAAFGGDPTsVTLFGESAGAAASV	wmGvpPHgyEiefiFglpl	vGvvkdEGsyFLvygapg	12
Mouse	NIAAFGGDPmsVTLFGESAGAAASV	wmGvpPHgyEiefiFglpl	vGvvkdEGsyFLvygvpG	13

non-polar side-chains) and obviously do not alter the ability of the two different B₁ esterases to sequester OPs. Amino acid sequences are now known for two B₁ esterases, a B₂ esterase, an A₂ esterase and a 'susceptible' B esterase. Amino acid differences between the 'susceptible' B esterase (or amino acid residues unique to this esterase) and the B₁ and B₂ sequences must contribute to the altered binding kinetics of the resistance-associated esterases for the OPs. The OPs are better inhibitors (substrates) of a number of resistance-associated A₂ and B₂ esterases when compared to the 'susceptible' A and B esterase from the *Cx quinquefasciatus* strain PelSS (Kettermann *et al.*, 1993; Karunaratne, 1994). At present, the residues involved in the increased binding of the OPs in the resistance-associated esterases are not known. Once the 'susceptible' A esterase amino acid sequence is known it will be possible to perform mutation studies on recombinant A and B esterases (resistant and susceptible) to deduce those residues important for insecticide binding. This in turn will lead to a greater understanding of the action of the esterases, aid new insecticide design and help to determine esterase structure. Residues around the active site histidine are less well conserved (TABLE 7.1), the histidine being the only conserved residue in all the sequences. Clearly, esterase function is not dependent on such specific residues around the histidine, although a number of residues are found in the majority of sequences. Even less well conserved, are the residues around the active site acid residue (glutamate or aspartate). In the majority of cases the acid residue is glutamate but the *D. melanogaster* esterases, Est6 and EstP have an active site aspartate. As for the residues surrounding the histidine, different combinations still preserve esterase function. It could be that different residues surrounding the histidine and acid of the active site triad play a greater part in determining the specificity of the esterases towards their substrates than those

residues surrounding the active site serine, since they are so well conserved and presumably essential for esterase activity.

The *Cx quinquefasciatus* A and B esterases form acyl intermediates with the oxon analogues of the OPs and the two products of the reaction are an organo-alcohol and organo-phosphoric acid. A successful OP insecticide must bind tightly to the active site of AChE and be very slowly released. The mechanism of the inhibition of AChE by OPs and carbamates is well documented (Corbett *et al.*, 1984). The active site serine of the AChE is phosphorylated by the OP (or carbamylated by the carbamate) and the subsequent hydrolysis of this group is very slow. The A and B esterases, presumably because their active sites are similar to that of AChE, also sequester a range of OPs and carbamates (Ketterman *et al.*, 1992; Karunaratne *et al.*, 1993a). Other substrates however, are rapidly hydrolysed by the A and B esterases and these include *p*-nitrophenyl acetate/hexanoate and α/β -naphthyl acetate (Ketterman *et al.*, 1992; Karunaratne *et al.*, 1993a). It is important to note that a single amino acid change in the active site region of AChE leads to very high OP resistance levels in insects carrying this mutation (INTRODUCTION 1.3.2.1). The amino acid change causes a decreased rate of binding of the OP (due to an alteration in the active site structure) and hence AChE is still able to hydrolyse acetylcholine (Fournier & Mutero, 1991; 1994; Pralavorio & Fournier, 1992; Fournier *et al.*, 1993). The structure of the OP resistance-related E4 enzyme from *M. persicae* must be slightly different from that of the *Cx quinquefasciatus* A and B esterases and AChE. The E4 enzyme has a dual role in resistance. As well as sequestering OPs and carbamates, the E4 esterase can also rapidly hydrolyse the pyrethroid, permethrin (Sawicki *et al.*, 1978; Devonshire & Moores, 1982). The interaction of the OPs and carbamates

with the E4 esterase active site must be different to that with permethrin which does not form a stable acyl-intermediate with the esterase but is rapidly hydrolysed.

The malathion carboxylesterases are unique to a number of malathion resistant insect strains, in that they rapidly hydrolyse malathion (cross-reactivity is also seen to phenthoate) but no other OPs or carbamates. Furthermore, the esterases are presumed not to be elevated in resistant strains (Matsumura & Voss, 1965; Hemingway, 1985; Ziegler *et al.*, 1987; Boddington, 1992; Whyard *et al.*, 1994). Malathion specificity is due to the esterase hydrolysing the carboxylic acid (not phosphoric acid) ester groups of the malathion (Matsumura & Brown, 1961b; Matsumura & Hogendijk, 1964). Malathion (and phenthoate) are unique amongst the commonly used OPs, in having carboxylic acid groups and this is why the malathion carboxylesterase is so specific. The structure of the active site of the malathion carboxylesterase is clearly different to that of the elevated *Cx quinquefasciatus* A and B esterases. The former rapidly hydrolyses malathion whilst the latter sequesters it. The active site structure of the malathion carboxylesterase leads to a high affinity for the carboxylic ester and it is quite unique in this respect since most carboxylesterases (including the *Cx quinquefasciatus* A and B esterases), have a preference for the phosphoric ester of the OPs. In the sheep blowfly *L. cuprina*, the malathion carboxylesterase from the resistant strain can not hydrolyse α -naphthyl acetate but the esterase from the susceptible strain can (Whyard *et al.*, 1994). The loss of ability to hydrolyse α -naphthyl acetate in the resistant strain esterase is associated with a greatly increased hydrolysis rate of malathion (activity is increased 45- to 50- fold over the susceptible esterase). This has also been seen in *Cx tarsalis* (Bigley & Plapp,

1962; Matsumara & Brown, 1963; Ziegler *et al.*, 1987) and *Anopheles* species (Hemingway, 1982; 1983; 1984; Lines *et al.*, 1984; Herath *et al.*, 1987). In early cases of OP resistance, a drop in general esterase activity (when compared with the susceptible) was often detected in malathion resistant insects (Oppenoorth & Van Asperen, 1960). The oxon analogue of malathion, malaoxon can inhibit the malathion carboxylesterase (Bigley & Plapp, 1962). Hence, the function of malathion carboxylesterase is to hydrolyse the malathion before it is oxidised to the highly active malaoxon by P450s (INTRODUCTION 1.3.1.2). The malathion carboxylesterase may be inhibited by other oxons also but since there is no detectable elevation of the esterase, this will not in itself provide resistance. It is probable that amino acid differences between the malathion carboxylesterases from the resistant and susceptible strains (most likely to be in the active site region), lead to the increased malathion hydrolysis (and associated loss of α -naphthyl acetate hydrolysis) in the resistance-associated esterase. Perhaps, as has been seen in resistance-associated, structurally altered target sites [AChE and the GABA-receptor (INTRODUCTION 1.3.3.1, 1.3.3.2)], a single amino acid change confers the kinetic change in malathion carboxylesterase activity.

7.2 ESTERASE GENE DUPLICATION

It is likely that the A and B esterase genes arose through a tandem duplication of an ancestral gene, since they are so similar (percentage identity of 49%, both 540 amino acids, both without N-terminal signal sequence, both share exon/intron boundaries) and lie so close together on the amplicon (about 2 kb apart). In *D. melanogaster*, a tandem duplication of an esterase gene is thought to have caused the appearance of Est6 and EstP (Collet *et al.*, 1990). Both the esterases have two exons (1387 and 248 bp) and an intron (51 and 56 bp respectively) and share 64%

nucleotide homology and 60% amino acid homology. Homologues of Est6 and EstP have also been found in *D. pseudoobscura* and *D. buzzatii* (Collet *et al.*, 1990). In the sibling species pair *D. mojavensis* and *D. arizonensis*, it has been suggested that a gene duplication gave rise to Est4 and Est5 (Zouros *et al.*, 1982), on the evidence of their close proximity to each other (0.16 recombination units apart at the 95% probability level). Elevated A and B esterases, as well as being characterized in *Cx quinquefasciatus*, have been identified in two further species, *Cx tarsalis* (Prabhaker *et al.*, 1987; Raymond *et al.*, 1989) and *Cx tritaeniorhynchus* (Takahashi & Yasutomi, 1987), indicating that the A/B esterase duplication occurred before *Culex* speciation, as for *D. melanogaster* Est6 and EstP. The closeness in proximity of the A and B esterase genes, certainly implies that they arose from a tandem duplication and their shared intron/exon boundaries add further weight to this hypothesis. In *M. persicae*, the esterase E4, involved in OP resistance, is very closely related to the other OP resistance related esterase, FE4. E4 has an open reading frame of 1656 nucleotides and in FE4, a single nucleotide substitution results in the loss of the stop codon and the subsequent increase of 36 nucleotides at the 3' end of the open reading frame (Field *et al.*, 1993). However, E4 and FE4 do not occur in the same insects and it is probable that an aphid line lost the stop codon from the E4 gene which then gave rise to the altered FE4 gene (Field *et al.*, 1993).

The TEM-R B₁ esterase gene (Mouches *et al.*, 1990) has three introns which are in identical positions in the PelRR A₂ esterase gene. There are three additional introns in the PelRR A₂ esterase gene and there is ongoing argument as to whether introns came early or late to eukaryotic genomes. The highly conserved enzyme, triosephosphate isomerase (TPI) is central to this issue. The 'introns

early' argument suggests that introns are as old as the genes themselves and the apparent correlation of many of the intron sites in plant, animal and fungal TPI genes, occurring between the boundaries of structural elements (Go, 1983) is evidence for the assembly of ancient proteins by exon shuffling (Gilbert *et al.*, 1986). In contrast, the 'introns late' view suggests that ancient genomes contained very few, if any, introns and they were inserted into pre-existing genes during the last billion years (Cavalier-Smith, 1985). Compact structural units in chicken lysozyme were found to be separated by introns, favouring the 'introns early' hypothesis (Go, 1983). Based on the exon shuffling hypothesis of the 'intron early' argument, it was proposed that an intron of the TPI gene, as yet undiscovered, would be found (Gilbert *et al.*, 1986). This novel intron site was found in the TPI gene from *Cx tarsalis*, in agreement with the 'intron early' hypothesis (Tittiger *et al.*, 1993). Since the *Culex* mosquitoes are thought to be primitive dipterans, the novel intron should also be observed in other mosquitoes, or their ancestors, such as Hymenoptera. Conversely, 36 intron positions have been assigned to the actin gene and analyzed with respect to the three-dimensional structure of actin, from a range of eukaryotes (Weber & Kabsch, 1994). Between 20 and 23 of these introns relate to the ends of secondary structural elements (favouring the 'introns early' hypothesis) but this observation can be explained as the result of a random insertion due to the high number of positions related to the termini of such structural elements (Weber & Kabsch, 1994). In the case of carboxylesterases, the AChE gene has been isolated from both *An. stephensi* (Hall & Malcolm, 1991) and *D. melanogaster* (Hall & Spierer, 1986). Although the size of the genes are greatly different (3 and 20 kb respectively) they both have 9 introns in exactly the same place. The *A. stephensi* AChE gene has an additional intron and as anophelines are thought to be ancestral to drosophilids, this adds weight to the

'introns early' hypothesis. The truth of the matter may well lie in between the two disparate hypotheses. Perhaps, initially, introns did separate structural elements but during the course of evolution, many of the introns have been lost and some gained in positions not associated with structural boundaries. In relation to the *Cx quinquefasciatus* A and B esterases, the 'intron early' hypothesis would suggest that the A esterase was ancestral to the B esterase and in the 'intron late' hypothesis the converse would be true. At present, no real conclusion can be drawn but if a primitive dipteran could be found with only an A or B esterase gene present, this would suggest that gene as being ancestral and in this particular case, provide evidence for one of the 'intron early/late' hypotheses. It would also add weight to the argument for a gene duplication giving rise to the A and B esterase genes.

7.3 ESTERASE GENE AMPLIFICATION

The exact mechanisms of gene amplification are poorly understood although a number of mechanisms have been put forward (Stark *et al.*, 1989). In both *Cx quinquefasciatus* and *M. persicae*, the esterase genes (A and B in *Cx quinquefasciatus* and E4 in *M. persicae*) associated with resistance are amplified (Field *et al.*, 1988; Raymond *et al.*, 1989). In the case of the *Cx quinquefasciatus* TEM-R B₁ esterase, the amplification is inherited through the germline (Nance *et al.*, 1990). This is undoubtedly the case for the other amplified B esterase genes, since the B esterase genes are allelic and likely to behave similarly. It is also probably true for the A esterase genes since the A₂ esterase gene is closely linked to the B₂ esterase gene. In *M. persicae*, tandem duplication of the gene(s), leads to an exponential increase (amplification) in the number of genes (1, 2, 4,

8 etc.), which is associated with an increase in the resistance of the aphids to OPs (Devonshire & Sawicki, 1979). In *Cx quinquefasciatus*, such studies have not been undertaken but successive rounds of exposure to insecticide does cause an increase in resistance (Mouches *et al.*, 1987; Peiris & Hemingway, 1990a), undoubtedly associated with an increase in gene copy number. The amplicon of the B₁ esterase from the *Cx quinquefasciatus* TEM-R strain has been studied. The amplicon contains two truncated copies of a transposable element (TE), named Juan (Mouches *et al.*, 1990) and since such elements are able to move around the genome, it was suggested that the presence of Juan may be linked to the amplification process (Mouches *et al.*, 1991).

Many full-length copies of Juan-C were found to be reiterated in the genome of *Cx pipiens* mosquitoes and were 4.48 kb long (Agarwal *et al.*, 1993). Analysis of the Juan sequence showed that it belonged to the long (greater than 500 bp) interspersed repetitive element (LINE) superfamily of retrotransposable ubiquitous DNA elements, which were initially found to be dispersed in the genome of humans, primates and rodents but have since been characterized in a variety of organisms including *Drosophila*, trypanosomes, *Neurospora* and *Zea mays* (Nocera & Sakaki, 1990). TEs are DNA sequences that are capable of movement (transposition) within the genome and are characteristic of the middle repetitive portion of the genome. Like all LINEs (members of the TEs), Juan-C has an adenosine-rich sequence at the 3' end of one strand and no long terminal repeat (LTR) sequences (typical of many TEs but always absent in LINEs) (Agarwal *et al.*, 1993). The adenosine-rich sequence was preceded by an AATAAA polyadenylation signal, as is typical of most genes transcribed by RNA polymerase II. Two long ORFs were located one after the other in the DNA

strand terminating with the adenosine-rich tail, both with possible initiator methionine codons (ATG). Typical of all LINEs, the ORFs coded for two proteins, one of which had a highly conserved cysteine-rich motif which is also found in nucleic acid binding proteins of retroviruses (Fawcett *et al.*, 1986; Priimagi *et al.*, 1988). The second protein had homology to the enzyme reverse transcriptase, essential for the replication of the RNA genome of all retroviruses (Champion *et al.*, 1992). In *Drosophila*, the LINE, *jockey*, is transcribed from an internal promoter by RNA polymerase II (Mizrokhi *et al.*, 1988). Location of the promoter within the LINE allows it to be preserved in the course of replication *via* the LINE's reverse transcriptase and accounts for the distribution of *jockey* and probably many other LINEs throughout the genome (Mizrokhi *et al.*, 1988).

In *D. melanogaster*, the phenomenon of inducer-reactive (I-R) hybrid dysgenesis, is caused by an inducer (*I*) factor, which is in fact a LINE (Bucheton *et al.*, 1984; Fawcett *et al.*, 1986). The *I* factor has since been found in other *Drosophila* species (Bucheton *et al.*, 1986) and the *I* factor LINE of *Drosophila teissieri* was shown to transpose in other *Drosophila* species, by its introduction into the germline of a reactive strain of *D. melanogaster* by *P* element-mediated transformation (Abad *et al.*, 1989). A homologue of the Juan-C LINE of *Cx pipiens*, Juan-A, has been found to be dispersed in the genome of three non-sibling *Aedes* mosquito species (Mouches *et al.*, 1992). Restriction enzyme digest patterns showed that there were many identical copies of the Juan-A element in the three *Aedes* species. This identity indicated that the transposons underwent a recent amplification in the strains analyzed and suggested that these elements had spread by horizontal transfer between the three non-sibling species. Since the Juan element in the TEM-R B₁ esterase amplicon is truncated, it is probably not

involved in the duplication of the amplicon in resistant strains. It has yet to be shown if the amplicon containing the A₂ and B₂ esterase genes from the OP resistant PelRR strain contains any LINEs. Their presence would enforce the hypothesis that they (the LINEs) were (or are) involved in the amplification process of the resistance-associated genes. To date however, no direct evidence has been reported of TE-associated resistance evolution in the field.

Gene amplification, as well as being inherited, can also occur at certain stages of the life cycle or be tissue specific. In mammalian tumours, for instance, amplification is tissue specific. Proto-oncogenes are frequently amplified in tumours and their amplification is one of the causes of tumour progression (for a recent review see Samarawickrema *et al.*, 1992). In *D. melanogaster*, there is a stage specific amplification of the major chorion genes, which form two chromosomal clusters. These clusters, prior to expression begin to replicate differently and by the end of choriogenesis, reach amplification levels of 50- to 100-fold (Komitopoulou *et al.*, 1986). Within the amplicon of the chorion gene, a *cis* acting amplification-control element, ACE-3, has been shown to be important but not essential (disruption of the ACE-3 element drastically reduced the level of gene amplification) in the amplification of the chorion genes (Swimmer *et al.*, 1989). Since both the *Cx quinquefasciatus* A and B esterases and the *M. persicae* E4 esterase are amplified it would be beneficial to sequence their amplicons to try and find common sequences which could be involved in the amplification process.

7.4 THE MIGRATION OF AMPLIFIED *CULEX QUINQUEFASCIATUS* ESTERASE GENES

The B₁ and the A₂/B₂ esterase phenotypes have been found in more than one continent (Georghiou & Pasteur, 1978; Villani *et al.*, 1983; Raymond *et al.*, 1987; Hemingway *et al.*, 1990; Wirth *et al.*, 1990; Bisset *et al.*, 1990; Peiris & Hemingway, 1993; Rivet *et al.*, 1994). Since the restriction enzyme digest patterns for the B₂ esterase genes are similar and patterns for 'susceptible' B esterase genes are highly variable, it has been suggested that very few amplification events have occurred and the esterase genes have then migrated around the world (Raymond *et al.*, 1991). The present study has shown identical genomic nucleotide sequence (including introns) for four partial B₂ esterase genes isolated from OP resistant *Cx quinquefasciatus* strains from around the world. The predicted partial amino acid sequence of the four B₂ esterases differed from that of a previously published B₂ esterase sequence (Raymond *et al.*, 1991) which suggests that an error had been made in the sequencing of the other B₂ esterase, if the migration of the same amplified gene has occurred. The scenario of a few amplification events is losing credibility, since the present study has isolated a second B₁ esterase gene (from the MRES *Cx quinquefasciatus* strain) and a further two B₂ esterases with different RFLP patterns have been found in Europe (Poirie *et al.*, 1992). Undoubtedly, as more amplified esterases are characterised, more individual amplification events will be discovered. Still, the A₂/B₂ phenotype is very common and the presence of a XDH gene sequence within the A₂/B₂ esterase amplicon (CHAPTER 5) implies that a further gene may also be amplified in strains carrying the A₂/B₂ genes. The presence of an amplified, functional, XDH gene could be advantageous to the mosquito and thus, those

mosquitoes carrying this gene would be selected for. The MRES *Cx quinquefasciatus* strain, as well as having an amplified B₁ esterase gene, also has an amplified A esterase gene (CHAPTER 5). The A esterase was not detected in the native PAGE of larval MRES homogenate since it migrates at the same speed as B₁ esterase. Following on from this, there is a possibility that the TEM-R *Cx quinquefasciatus* strain (and other strains with B₁ esterase) also has an amplified A esterase gene. Since the A₂ and B₂ esterase genes sit next to each other on the amplicon, it is more than likely that a similar situation exists in the MRES strain and even in TEM-R. Surprisingly, no amplified B esterase was found to be associated with the elevated A₁ esterase of *Cx pipiens* (Raymond *et al.*, 1989). However, since the elevation of the A₁ esterase is low, the similarly low amplification of an associated B esterase gene could have been overlooked. Now an A esterase DNA probe is available, it will be easy to detect amplification of the A esterase gene in strains thought to carry only an amplified B esterase gene.

7.5 FURTHER WORK

The present study has shown that both the *Cx quinquefasciatus* A₂ and B₂ esterases involved in OP resistance are co-amplified. Further work needs to be carried out to determine if the co-amplification of A and B esterases is common to all resistant strains where an A or B esterase was initially identified [as is the case of the strains TEM-R and MRES (where only a B₁ esterase was initially characterised)]. Since the A and B esterases are so similar (percentage identity of 47%), it is likely they arose from a gene duplication and the presence of the A and B esterase genes in close proximity in an insecticide susceptible strain would help confirm this hypothesis and also show that the genes were not in some way transposed onto the same amplicon in the OP resistant strains.

A partial XDH gene sequence has been found on the A₂/B₂ esterase amplicon. If the XDH gene within the amplicon is functional it may contribute an additional benefit to the resistant insect. This would in turn explain why the A₂/B₂ esterase phenotype is so common. Therefore it will be interesting to see if any of the other amplicons contain this gene and whether the gene is functional in the A₂/B₂ esterase amplicon.

Initial expression studies of the A₂ esterase in the baculovirus system are promising. The isolation of fully functional recombinant A₂ esterase will hopefully be quicker and cheaper than purification from whole larvae. A larger supply of the esterase could lead to crystallisation of the protein and the elucidation of its three-dimensional structure. This will aid in the understanding of how the esterase sequesters the OPs and also aid in the design of new insecticides to overcome this resistance mechanism.

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APPENDIX

PUBLICATIONS, ABSTRACTS AND PAPERS FROM MEETINGS ALREADY ARISING FROM THIS STUDY:

- 1) Vaughan, A., Rodriguez, M. & Hemingway, J. (1995). The independent gene amplification of indistinguishable esterase B electromorphs from the insecticide resistant mosquito *Culex quinquefasciatus*. *Biochem. J.* 305, 651-658.
- 2) Vaughan, A. and Hemingway, J. (1995). Mosquito carboxylesterase Est α 2 (A₂). Cloning and sequencing of the full length cDNA for a major insecticide resistance gene found worldwide in the mosquito *Culex quinquefasciatus*. *J. Biol. Chem.* (accepted May 1995).
- 3) Vaughan, A., Ketterman, A.J. & Hemingway, J. (1993). Comparisons of nucleic acid sequences of esterases from resistant and susceptible strains of *Culex quinquefasciatus*. *Biochem. Soc. Trans.* 21, 481. (abstract).
- 4) Vaughan, A., Rodriguez, M., Karunaratne, S.H.P.P., Small, G.J., Ketterman, A.J., Chadee, D. & Hemingway, J. (1994). Characterization of non-specific esterases in *Culex quinquefasciatus* insecticide resistance. *8th IUPAC Cong. Pestic. Chem.* (abstract).
- 5) Hemingway, J., Ketterman, A.J., Karunaratne, S.H.P.P., Jayawardena, K.G.I. & Vaughan, A. (1993). Amplified esterases A₂ and B₂ has resistance occurred once or several times? *Proc. 1st Int. Cong. Ins. Pests Urban Envir.* 1, 319-328. (meeting).

